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(54) Title: USE OF PROTEASE INHIBITORS TO MODULATE CELLULAR PATHWAYS, IMMUNITY AND THERAPIES ASSOCIATED THEREWITH

(57) Abstract

The present invention is directed to the use of protease inhibitors, especially HIV protease, proteasome, serine protease and cysteine protease inhibitors to modulate cellular pathways such as those involved in cell activation, metabolism, proliferation, differentiation, maturation, cycle, and death. This is useful especially in the context of cancer treatment, allergy, vaccines, autoimmune disorder, inflammation, transplant, burn, trauma, acute ischemia, stroke, aging, wasting syndrome, and infectious conditions.

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USE OF PROTEASE INHIBITORS TO MODULATE CELLULAR PATHWAYS, IMMUNITY AND THERAPIES ASSOCIATED THEREWITH

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention hinges on the discovery that molecules that target proteases, especially viral or microbial proteases such as viral protease inhibitors, or proteasome inhibitors, may be used to modulate cellular pathways that regulate cell metabolism, cell activation, cell proliferation, cell differentiation, cell mutation, cell cycle and cell death. Because of the myriad of cellular pathways that may be modulated according to the invention using such inhibitors, the subject methods have numerous applications in the fields of human therapy and diagnosis, agriculture and veterinary medicine.

More specifically, the present invention relates to the use of HIV protease inhibitors or proteasome inhibitors to modulate cellular pathways. Especially, the invention relates to a method for enhancing the efficacy of vaccines and a method for modulating the immune system as well as modulating cellular immunity, cellular metabolism, cell proliferation, cell differentiation, cell maturation, cell cycle, and cell activation using HIV protease inhibitors, or inhibitors of proteasomes.

2. Description of the Related Art

There is compelling evidence implicating both humoral and T cell-mediated immune mechanisms in the prevention and control of Human Immunodeficiency Virus type I (HIV-1) (Emini et al., *Journal of Virology*, 64:3674-8, 1990; Emini et. al., *Nature*, 355:728-30, 1992; Conley et. al., *Journal of Virology*, 70:6751-8, 1996; Haigwood et. al., *Immunology Letters*, 51:107-14, 1996; Putkonen et. al., *Nature*, 352:436-8, 1991; Prince et. al., *AIDS Research & Human Retroviruses,*

7:971-3, 1991; Parren et. al., *AIDS*, 9:F1-6, 1995; Murthy et. al., *Immunology Letters*, 51:121-124, 1996; Koup et. al., *Blood*, 73:1909-14, 1989; Koup et. al., *Journal of Experimental Medicine,* 174:1593-600, 1991; Koup et. al., *Journal of Virology*, 68:4650-5, 1994; Harrer et. al., *Journal of Immunology*, 156:2616-2623, 1996; Harrer et. al., *AIDS Research & Human Retroviruses*, 12:585-592, 1996). Data demonstrating that monoclonal and polyclonal antibodies against HIV-1 or Simian Immunodeficiency Virus (SIV) transfer protection against homologous challenge in animal models established direct evidence for a humoral mechanism of protection (Emini et al., *Journal of Virology*, 64:3674-8, 1990; Emini et. al., *Nature*, 355:728-30, 1992; Conley et. al., *Journal of Virology*, 70:6751-8, 1996; Haigwood et. al., *Immunology Letters*, 51:107-14, 1996; Putkonen et. al., *Nature*, 352:436-8, 1991; Prince et. al., *AIDS Research & Human Retroviruses,* 7:971-3, 1991; Parren et. al., *AIDS*, 9:F1-6, 1995; Murthy et. al., *Immunology Letters*, 51:121-124, 1996). The case for cellular mechanisms, on the other hand, is indirect and stems from epidemiological observations correlating the decline of HIV-1 viremia in infected individuals with an increase in cytotoxic T lymphocytes (CTLs) against HIV-1 (Koup et. al., *Blood*, 73:1909-14, 1989; Koup et. al., *Journal of Experimental Medicine,* 174:1593-600, 1991; Koup et. al., *Journal of Virology*, 68:4650-5, 1994; Harrer et. al., *Journal of Immunology*, 156:2616-2623, 1996; Harrer et. al., *AIDS Research & Human Retroviruses*, 12:585-592, 1996; Borrow et. al., *Journal of Virology*, 68:6103-10, 1994). In addition, epidemiological evidence suggests that HIV-1 infected long-term non-progressors develop higher levels of HIV-1-specific CD8+ T cells that secrete an HIV-1 suppressive factor, than rapid progressors (Walker et. al., *Science*, 234:1563-6, 1986; Walker et. al., *Cellular Immunology*, 119:470-5, 1989; Walker et. al., *Immunology*, 66:628-30, 1989; Wiviott et. al., *Cellular Immunology*, 128:628-34, 1990; Castro et. al., *Cellular Immunology*, 132:246-55, 1991; Walker et. al., *Journal of Virology*, 65:5921-7, 1991; Walker et. al., *Cellular Immunology*,

137:420-8, 1991; Hsueh et. al., *Cellular Immunology*, 159:271-9, 1994; Paxton et. al., *Nature Medicine*, 2:412-417, 1996), which has now been shown to be mediated at least in part by β chemokines (Cocchi et. al., *Science*, 270:1811-5, 1995). On the whole, these lines of evidence strongly suggest that broadly neutralizing antibody either alone or in conjunction with chemokine secreting T cells and CTLs, will convey broad protection against HIV-1 in humans.

A number of groups have reported vaccine-induced protection against HIV-1, HIV-2 or SIV in non-human primates (Girard et. al., *PNAS*, 88:542-6, 1991; Putkonen et. al., *AIDS Research & Human Retroviruses*, 7:271-7, 1991; Marx et. al., *Science*, 260:1323-7, 1993; Putkonen et. al., *Journal of Acquired Immune Deficiency Syndromes*, 7:551-9, 1994; Franchini et. al., *AIDS Research & Human Retroviruses*, 11:909-20, 1995; Abimiku et. al., *Nature Medicine*, 1:321-9, 1995; Lehner et. al., *Nature Medicine*, 2:767-75, 1996; Boyer et. al., *Journal of Medical Primatology*, 25:242-250, 1996; Berman et. al., *Journal of Infectious Diseases*, 173:52-59, 1996; Boyer et. al., *Nature Medicine*, 3:526-532, 1997). The focus of the vast majority of these early vaccine studies was on systemic immune responses and parenteral virus challenges (Girard et. al., *PNAS*, 88:542-6, 1991; Putkonen et. al., *AIDS Research & Human Retroviruses*, 7:271-7, 1991; Marx et. al., *Science*, 260:1323-7, 1993; Putkonen et. al., *Journal of Acquired Immune Deficiency Syndromes*, 7:551-9, 1994; Franchini et. al., *AIDS Research & Human Retroviruses*, 11:909-20, 1995; Abimiku et. al., [see comments] *Nature Medicine*, 1:321-9, 1995; Lehner et. al., *Nature Medicine*, 2:767-75, 1996; Boyer et. al., *Journal of Medical Primatology*, 25:242-250, 1996; Berman et. al., *Journal of Infectious Diseases*, 173:52-59, 1996; Boyer et. al., *Nature Medicine*, 3:526-532, 1997; Myagkikh et. al., *AIDS Research & Human Retroviruses*, 12:985-992, 1996). This test scheme was convenient for early studies and may model transmission when it occurs via parenteral inoculation. However, the majority of HIV-1 vaccines resulting from these studies elicited

unsatisfactory responses. Awareness of these facts provided the impetus behind a growing effort to develop a practical way to enhance the efficacy of HIV-1 vaccines. One of the current challenges to the development of a protective vaccine has been the difficulty in defining the correlates of protection. Irrespective of mechanism, whether by humoral or cellular responses, viral suppressive factors, or the combination of all, what seems to be needed is to generate an immune response that is more effective than that induced by the natural HIV infection or conventional vaccination. A successful vaccine takes more than having the right antigen presented at the relevant anatomic localization. The immune environment within an organism constituted by multiple factors including the interplay of cytokines and regulatory signals determines the effectiveness of an immune response as well.

Other challenges to generating a highly effective immune response in an HIV infected person are the alterations in immune function caused by HIV gene products. Illustrated in a simplified way: too many immune cells are driven into a stage of activation that correlates with a high susceptibility to HIV infection ("chronic activation"), and specific effector cells and their precursors, on the other hand, are paralyzed in anergy or highly susceptible to cell death by apoptosis. In that context, it is most important to note that immune cells with the highest specificity (as defined by avidity) for their targets are much more susceptible to being driven into apoptosis than cells with lower avidity (Berzofsky, J., communicated at the Annual Meeting of the Institute of Human Virology, Baltimore, 8-24-98). Thus, in HIV infection, the most effective immune response may be lost before the "mission" is executed. Cytokine "disturbances," such as increased levels of type-I and -II interferons, TNF-alpha, TRAF, Fas-ligand and the like or decreased levels of IL-2, IL-12, GM-CSF and the like, coincide with immune dysfunction (Fauci et al., *Science*, 262: 1011-1018, 1993). Therefore, it is a prerequisite to normalize the cytokine "environment," and lower the

exceedingly high levels of anergy and apoptosis to vaccinate successfully an immune compromised individual.

Currently, highly active anti-retroviral therapy (HAART) is used to treat HIV-infected people can halt disease progression and partially restore immune functions. However, the frequent development of drug resistance to HAART therapy limits its window of effectiveness. In addition, HAART does not efficiently eliminate the HIV reservoir in persistent and latently infected cells. It has become evident that this reservoir of chronic, latently infected cells, although not necessarily contributing substantially to the plasma viral load, nevertheless plays a significant role in sustaining the infection.

Consequently, a successful therapy or vaccine must be aimed at both blocking HIV replication, as well as eliminating the viral reservoir. One strategy is to significantly enhance HIV-specific immune responses to the "super natural" level such that HIV replication is blocked and latently infected cells are eliminated. This will, however, require enhancing the immune response beyond the level of that obtained by HIV infection itself. Thus, therapeutic vaccines have to overcome the limitations of immune responses to natural HIV infection.

Accordingly, there is a legitimate need in the art to develop a practical and effective method to enhance vaccines, in particular, HIV-1 vaccines. Further, there is a need in the art for a method to modulate the activation of cells of the immune system and to inhibit cells of the immune system from undergoing apoptosis or becoming anergic. In addition, there is a need in the art for a method to modulate cytokines of the immune system.

Currently, the most effective treatment available for HIV-1 infection is a three-drug regimen containing a protease inhibitor and two nucleoside analogs. (Richman, D.D. Science 272:1886-1888 (1996); Danner et al, N. Engl. J. Med. 333:1528-1533 (1995); and Kempf et al, Proc. Natl. Acad. Sci., USA 92: 2487-2488 (1995)). Various inhibitors of HIV-1 protein have been successfully used

for the treatment of HIV-1 infected patients and AIDS disease. The use of such inhibitors has been found to profoundly reduce AIDS-related mortality. (*See*, e.g., CDC: Update: Trends in AIDS incidence, detection, and prevention; United States, MMWR 46(8):163-173 (1997); Palella et al, N. Engl. J. Med. 338:853-850 (1998); Moore et al, Pharmacoeconomics 10(2):109-113 (1996); Keiser et al, J. Acquir, *Immun. Defic. Syndr. Hum. Retrovirol.* 20(1):28-33 (1999); and Bozzette et al, N. Engl. J. Med. 339(26):1897-1904 (1998)).

It is also known that protease inhibitors can have a significant effect on opportunistic infections with HIV-1 infection and AIDS. While in some instances the use of protease inhibitors has a beneficial effect on the incidence and resolution of opportunistic infections, paradoxically the use of such inhibitors has in some instances had an adverse effect, i.e., resulted in worsening or increased incidence of such infections. (See, e.g., Jacobsen et al, Lancet 349:1443-1445 (1997); Michelet et al, AIDS 12:1815-1822 (12998); Rodriguez-Rosado et al, Antiviral Therapy 3:229-231 (1998); Narita et al, *Am. J. Respor. Crit. Care Med.* 158:157-161 (1998); Clifford et al, Neurology 52:623-625 (1999); Arrizabulaga et al: Incidence and risk factors for developing CMV retinitis in HIV-infected patients receiving protease inhibitor therapy [Abstract 251] 6th *Conference on Retrovirus and Opportunistic Infections*, Chicago, Ill (1999); Karavellas et al, J. Infect. Dis. 179:697-700 (1999).)

Further, while the use of inhibitors of HIV-1 protease for the treatment of HIV-1 infection is well established, the actual mechanism(s) by which such inhibitors are effective remains poorly understood. In particular, the extent of immunological recovery after protease inhibitor treatment is the subject of debate.

Unexpectedly, in some patients, virus titers are not drastically altered but CD4+ T-cells increase remarkably (Perrin et al, *Science* 280:1871-1873 (1998)), whereas in some other patients, transient reactivation of other persistent viruses after treatment has been observed (Jacobsen et al, *Lancet* 349:1443-1495 (1997);

Carr et al, *Lancet* 349:993-996 (1997); and Rutschmann et al, *J. Infect. Dis.* 177:783-785 (1998)).

It is known that cytotoxic T lymphocytes (CTLs) play an important role in early defense, especially against several non-cytopathic infections and against HIV-1. Related thereto, it has been hypothesized that HIV-1 protease inhibitors may elicit effects other than interfering with HIV replication.

Recently, the possible effect of a particular HIV-1 protease inhibitor, Ritonavir, on immune responses in general and on *in vivo* CTL activity in particular, was reported in the literature. (Andre et al, *Proc. Natl. Acad. Sci., USA* 95:13120-13124 (1998)). This was investigated in mice infected with lymphocytic choriomeningitis virus (LCMV), a non-cytopathic arenavirus in which CTLs are responsible both for the initial viral control and for virus-induced immunopathological disease. The researchers speculated based on their results that Ritonavir may modulate proteasome activity and major histocompatibility complex (MHC) class I-restricted presentation of several LMCV and MART-1 epitopes *in vivo* and *in vitro* and that these observations may help to explain some of the observations made in AIDS patients undergoing highly active anti-retroviral therapy.

Also, it was recently reported that cell-mediated immune function improved during potent anti-HIV therapy with Ritonavir and Saquinavir. (Angel et al, *J. Infect. Dis.* 177:898-904 (1998)). However, notwithstanding what has been reported to date, and further notwithstanding the well established use of HIV-1 protease inhibitors for the treatment of HIV-1 infection, the actual effects of HIV-1 protease inhibitors on cellular and immunological responses remain poorly understood.

SUMMARY AND OBJECTS OF THE INVENTION

It is an object of the invention to use an HIV-1 protease inhibitor or a proteasome inhibitor as a model to ascertain the effects of molecules that target proteases, particularly protease inhibitors, and more particularly inhibitors of viral or microbial proteases, on cellular pathways.

It is a more specific object of the invention to an use HIV-1 protease inhibitor or a proteasome inhibitor as a model to ascertain the effects of protease inhibitor, especially microbial or viral protease inhibitors such as cysteine or serine protease inhibitors on cellular pathways that regulate cell-metabolism, -activation, -proliferation, -differentiation, -maturation, -cycle and -death.

It is also an object of the invention to use an HIV-1 protease inhibitor or proteasome inhibitor as a model to evaluate the effects of protease inhibitors on systemic metabolic changes such as increases in blood sugar, lipids, and decreases in protein wasting.

It is another object of the invention to provide novel treatments involving the administration of at least one protease inhibitor, e.g., an HIV protease, proteasome, serine protease or cysteine protease inhibitor, to a human or animal in need of such treatment, wherein such treatment is effected to modulate at least one cellular pathway that regulates cell-metabolism, -activation, -proliferation, -differentiation, -maturation, -cycle or -death.

It is another object of the invention to provide novel treatments involving the administration of at least one protease inhibitor, e.g., an HIV protease, proteasome, serine protease, or cysteine protease inhibitor, to a human or animal in need of such treatment that is effected in order to modulate systemic metabolic changes such as sugar, lipids, and protein metabolism.

It is a more specific object of the invention to provide a novel method to modulate and/or enhance the immune system utilizing compounds or molecules that target, bind to or interact with a protease, e.g., a microbial or viral protease,

such as HIV-1 protease inhibitor, proteasome inhibitor, serine protease inhibitor, or cysteine protease inhibitor.

It is another object of the invention to provide a novel method to enhance prophylactic and therapeutic vaccinations by additionally administering to the vaccinated subject at least one protease inhibitor, such as an HIV-1 protease or proteasome inhibitor, prior, concurrent, and/or after vaccination.

It is still another object of the invention to provide a novel method to increase memory and naïve cells of the immune system using a protease inhibitor such as an HIV-1 protease or proteasome inhibitor.

It is yet another object of the invention to provide a novel method to effect specific types of immune responses, such as Th1 or Th2 responses using a protease inhibitor, e.g., an HIV-1 protease or proteasome inhibitor.

It is a more specific object of the invention to provide a novel method to enhance HIV-1 vaccinations using a protease inhibitor, e.g., an HIV-1 protease, proteasome, cysteine protease, or serine protease inhibitor.

It is another object of the invention to provide a novel method for treating HIV-2 infection by administering a protease inhibitor, e.g., an HIV-1 protease, proteasome, cysteine protease, or serine protease inhibitor.

It is an object of the invention to provide a novel method to modulate the activation of cells and cytokines of the immune system as well as different aspects of cellular metabolism by the administration of a protease inhibitor, e.g., an HIV-1 protease, proteasome, cysteine protease, or serine protease inhibitor.

It is another object of the invention to provide a novel method to modulate T-cell subsets and lymphocyte function by the administration of a protease inhibitor, e.g., an HIV-1 protease, proteasome inhibitor, cysteine protease, or serine protease inhibitor.

It is still another object of the invention to provide a novel method to protect cells or tissue from different kinds of death signals by the administration

of a protease inhibitor, e.g., an HIV-1 protease, proteasome inhibitor, cysteine protease, and serine protease inhibitor.

It is another object of the invention to provide a novel method to inhibit cells of the immune system from undergoing apoptosis or becoming anergic by the administration of a protease inhibitor, e.g., an HIV-1 protease, proteasome, serine protease, or cysteine protease inhibitor.

It is yet another object of the invention to provide a novel method for treating diseases or conditions relating to the immune system by the administration of a protease inhibitor, e.g., an HIV-1 protease, proteasome, cysteine protease, or serine protease inhibitor.

It is still another object of the invention to provide a novel method to effect changes in immune cell proliferative capacity and cell function by the administration of a protease inhibitor, e.g., an HIV protease, proteasome, cysteine protease, or serine protease inhibitor.

It is another object of the invention to provide a novel method for cancer treatment and/or prophylaxis by the administration of a protease inhibitor, e.g., an HIV protease, proteasome, cysteine protease, or serine protease inhibitor.

It is yet another object of the invention to provide a novel method for the treatment and/or prophylaxis of inflammatory diseases or conditions by the administration of a protease inhibitor, e.g., an HIV protease, proteasome, serine protease, or cysteine protease inhibitor.

It is still another object of the invention to provide a novel method for the treatment of immuno-compromised hosts by the administration of a protease inhibitor, e.g., an HIV protease, proteasome, cysteine protease, or serine protease inhibitor.

It is another object of the invention to provide a novel method to modulate immune cell and non-immune cell proliferation by the administration of a protease

inhibitor, e.g., an HIV protease, proteasome, cysteine protease, or serine protease inhibitor.

It is yet another object of the invention to provide a novel method to control tissue generation and degradation by the administration of a protease inhibitor, e.g., an HIV protease, protease, cysteine protease, or serine protease inhibitor.

It is still another object of the invention to provide a novel method for the therapeutic modification of cell and tissue maintenance and cell homeostasis by the administration of a protease inhibitor, e.g., an HIV protease, proteasome, cysteine protease, or serine protease inhibitor.

It is still yet another object of the invention to provide a novel method to treat infectious diseases by the administration of a protease inhibitor, e.g., HIV protease, proteasome, serine protease, or cysteine protease inhibitor.

It is another object of the invention to provide a novel method for decreasing rejection responses in hosts receiving transplants and/or grafts by the administration of a protease inhibitor, or an HIV protease, proteasome, serine protease, or cysteine protease inhibitor.

It is a more specific object of the invention to provide novel methods for modulating at least one of inflammation, the effects of aging, anti-wasting, acute ischemia, neovascularization, apoptosis, anti-cancer cell responses, autoimmune diseases, transplantation, by the administration of an effective amount of at least one protease inhibitor, e.g., an HIV-1 protease inhibitor, proteasome inhibitor, serine protease inhibitor, or cysteine protease inhibitor.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1:

Ritonavir related changes in susceptibility to cell death. Preactivated PBMC were exposed to IFN-alpha (300IU/ml), IFN-gamma (30ng/ml), and TNF (10ng/ml), or their combinations for 3 days in the presence or absence of Ritonavir. After three days, CD95 agonistic mAb CH11 (200ng/ml) was added for 16h. Cell viability as

assessed by standardized WST-1 assays in quadruplicate cultures is depicted (Means ± SD). Differences between Ritonavir treated and untreated groups are statistically significant (paired t-Test, $p<0.05$). One representative experiment is illustrated out of four performed with similar results from different cell donors.

Figure 2:

HIV Protease Inhibitor related changes in susceptibility to cell death. Preactivated PBMCs were exposed to IFN-alpha (50 ng/mL), IFN-gamma (50 ng/mL) and TNF-alpha (50 ng/mL) in combination in the presence or absence of HIV Protease Inhibitors at low, medium and high concentrations, before adding CD95 agonistic monoclonal antibody, CH11. Cell viability was assessed by standardized WST-1 assays. Caspase 1 (ICE) Inhibitor was added at 100 micromolar (high), 10 micromolar (medium) and 1 micromolar (low); Saquinavir, Nelfinavir, Ritonavir and Indinavir were used at 100 nM (high), 10 nM (medium), 1 nM (low) for treatments.

Figure 3:

HIV Protease Inhibitor related changes in susceptibility to cell death. Preactivated PBMCs were exposed to IFN-alpha (50 ng/mL), IFN-gamma (50 ng/mL) and TNF-alpha (50 ng/mL) in combination in the presence or absence of HIV Protease Inhibitors at low, medium and high concentrations, before adding CD95 agonistic monoclonal antibody, CH11. Apoptosis Index was assessed by standardized TUNEL assays. Caspase 1 (ICE) Inhibitor was added at 100 micromolar (high), 10 micromolar (medium) and 1 micromolar (low); Saquinavir, Nelfinavir, Ritonavir and Indinavir were used at 100 nM (high), 10 nM (medium), 1 nM (low) for treatments.

Figure 4:

Increased colony forming capacity in bone marrow derived cells. Increase in colony forming capacity in bone marrow derived cells was observed under ritonavir treatment in vitro, as compared to other protease inhibitors (ICE-I =

caspase-1 inhibitor, MG132 = reversible proteasome inhibitor). The effect on increase in colony formation extends to all hematopoietic lineages.

Figure 5:

Mice treated with cytoxan in the presence or absence of Ritonavir. Mice treated with cytoxan for one week in the presence (left panel) or absence (right panel) of PR-I (Ritonavir) treatment for three weeks. Peripheral white blood cell counts (WBC) were evaluated weekly. This figure illustrates the growth promoting effects of the protease inhibitor and, it also indicates that the drug leads to a faster recovery after exposure to toxins.

Figures 6a-6d:

The effects of Ritonavir on immune responses. These figures evaluate the effects of systemic treatments of Ritonavir as immune adjuvant (6a) and local co-injection of Ritonavir as adjuvant (6b) on specific humoral (antibody) immune responses induced by immunizations. It can be seen from these results that an increased amount of neutralizing antibodies were detected in the serum of mice (group of 5) that received co-treatment with the PR-I (Ritonavir). Higher antibody titers that were induced by Ritonavir treatment were functional in that the antibodies had a greater neutralization of HIV-1 as measured by infection of indicator cells (6c and 6d). Data from pooled serum of 5 mice per treatment group. Results have been reproduced twice in independent experiments, in one of which ovalbumin was used as antigen. Mice that received proteasome inhibitor treatments during immunization with recombinant HIV gp 140 developed significantly higher titer of specific antibodies than the control group as measured by ELISA, 30 days after a single injection of Ag in IFA or alum.

Figure 7:

Growth promoting effects of Ritonavir on PBMC. Results show viability after 15 days of treated as compared with untreated cultures. Increased cell numbers, as compared to untreated controls, were measured in cultures treated with lower

concentrations (lower toxicity) of Ritonavir, detectable at concentrations as low as 0.5 nmol/l. These results were confirmed by cell counts using Trypan blue exclusion. Data were obtained from triplicate cultures and two independent, but similar experiments were combined. Standard deviation (SD) < 10% of mean values.

Figure 8:

Decrease in HIV transgene expression by Ritonavir. HIV transgene expression as measured in RNA extracts from tail clippings, that were probed with full length HIV-1 probes (Northern blot), was decreased by Ritonavir treatment.

Figure 9:

Dose-dependent inhibition of cell death by Ritonavir. Pretreated, activated PBMC were incubated with the indicated concentrations of Ritonavir for 3 days and CD95 was triggered by adding of various concentrations of the agonistic mAb CH11 for 2 days. Viability determined in standardized WST-1 assays was significantly increased in Ritonavir treated PBMC cultures compared to untreated controls at CH11 concentrations ranging from 50 ng/mL to 400 ng/mL. Data obtained from duplicate cultures are shown (Means ± SD) that are representative of three experiments performed using PBMC derived from 3 different donors.

Figures 10a - 10e:

Analysis of apoptosis markers in Ritonavir treated, pre-activated PBMC cultures. Viable CD3+ cells were gated and histogram overlays are shown for:

A) Intracellular Casp1 (ICE) staining; Ritonavir treated cells (10nM, slim-lined curve) are compared to untreated controls (bold line). Controls for non-specific stain (dotted curve) were obtained by competition with specific peptides to anti-Casp1 antibodies. **B)** Cell surface staining with Annexin V; intensity of staining in Ritonavir treated T-cells (slim line) is compared to that of untreated cells (bold). **C)** Cell membrane staining for CD95 (Fas)-ligand in CD3+ CD4+ cells; Ritonavir treated cells (line) are compared with untreated cells (bold). A

decrease in T-cell surface Fas-ligand expression was determined by Flow cytometry. **D)** It can be seen that CD95-ligand expression on T-cells was markedly decreased in cultures treated with 10 nM Ritonavir. These effects were detectable in both the CD4+ and CD8+ T-cell subset, but more pronounced in the CD4+ cell population. Ritonavir mediated decreases in Fas-ligand protein-content in lysates of PBMC were measured by Western blot and densitometrical quantification of specific protein bands. **E)** This figure depicts a Western blot of fas-ligand and other proteins. It can be seen that when Ritonavir decreases proteasome activity, the re is a subsequent increase in ubiquitinated proteins (which are normally processed by proteasome complexes) and a decrease in fas-ligand expression.

Figures 11a and 11b:

Effect of Ritonavir on caspase-3 activity. **A)** PBMC pre-activated with anti-CD3 mAb were triggered to apoptosis by the CD95 agonistic mAb CH11. Caspase-3 enzymatic activity was measured in cytosolic extracts from cells at the indicated time points using a fluorometric assay. Induction of caspase-3 activity by CD95 mediated signaling was inhibited by the addition of 1umol/l Ritonavir to the cultures. One representative experiment is shown, out of three performed with cells derived from three different HIV sero-negative donors. Samples were measured in duplicate and mean values (± standard deviation) are depicted. **B)** This figure contains the results of an experiment which showed that Ritonavir treatment increased caspase-3 activity and apoptotic death in immortalized (tumor) cells, specifically human promonocytic leukemia (U937) cells. Similar results were obtained when HL60, MOLT3, and other tumor cell lines were treated with Ritonavir, thus indicating a selective-for-tumor-cells increase of apoptosis that is mediated by PI (caspase-3 assay, PharMingen, was performed following the manufacturer's recommendations).

Figures 12:

Differential regulation of inflammatory cytokines and beta-chemokines. The differential regulation of inflammatory cytokines and beta-chemokines is dose-dependent and reversible, as assessed by secreted protein levels. Dose- and time dependent inhibition of TNF release, IFN-gamma, IL-10, IL-4, IL-1β and IL-6 in PBMC cultures by Ritonavir. PBMC were activated with anti-CD3 mAb (0.5 ug/ml) in the presence of the indicated concentrations of Ritonavir. At the indicated time points, supernatants from cultures were collected and TNF was quantified by ELISA. Each data point represents the mean value of duplicate determinations (SD < 15%). One experiment out of two performed, with similar results, is shown.

Figure 13:

Effect of Ritonavir on TNF induction in elutriated monocytes. Culture supernatants were collected after 16h of activation with LPS (10ng/ml) in the presence of the indicated concentrations of Ritonavir and assessed by ELISA. Data (Means ± SD) from one representative experiment (performed in triplicate cultures) is illustrated. Similar results were obtained with elutriated monocytes derived from 4 different individuals.

Figure 14:

Differential expression of cytokine and chemokine mRNA. Differential expression of cytokine and chemokine mRNA, as compared to the internal standard (GAPDH mRNA) was induced by PI treatment at 1micromol/l in activated monocyte cultures. Rnase protection assays revealed also a reversibility of PI related changes in mRNA expression. The following cytokines were analyzed: MIP-1α, MCP-1, RANTES, TNF, and IL-1β.

Figure 15:

The effects of Ritonavir on NFkB. This figure contains the results of Luciferase reporter gene transfection (Lipofectamine system, Gibco BRL) experiments in

COS cells and KSIMM cells. Expression of the reporter gene that contains an NFkB responsible element (Promega) was shown to be inhibited by Ritonavir treatment in a dose and time dependent fashion, wherein NFkB activation was induced by TNF (10 ng/ml).

Figures 16a and 16b

The effects of Ritonavir on cell surface receptors. These figures contain the results of an experiment that assessed flow cytometry surface receptors that are involved in endothelial cell activation and function. It can be seen that there was a dose-dependent inhibition by Ritonavir of integrin receptor alphaV-beta3, ICAM-1, VCAM-1 and E-selectin.

Figure 17:

The effects of Ritonavir on HIV transgenic mice. These figures contain the results of experiments wherein Ritonavir was administered to HIV transgenic mice, an accepted model for HIV associated pathologies. (The HIV-1 Tg-26 transgenic mouse expresses an HIV genome that lacks the HIV protease gene (Dickie et al., *Virology*, 185:109-19, 1991). Because HIV protease is absent, any effects of the HIV protease inhibitor Ritonavir on pathologies displayed by this animal model reflects side-effects of the drug.) It can be seen that skin lesions in Tg-26 mice healed after 2 weeks of treatment (30 mg/kg daily, comparable to human therapeutic doses).

Figure 18:

Histological results of mice treated with Ritonavir. These figures contain histological results of mice treated with Ritonavir. These results show that organ abnormalities and signs of lymph-infiltration declined after treatment with Ritonavir for three weeks. Tissue biopsies of skin, spleen and kidney are depicted in histological preparations.

Figures 19a and 19b:

The effects of Ritonavir on irradiated mice. This figure contains the results of an experiment wherein FVB\N mice (n=6) were pre-treated for 3 days with Ritonavir (30 mg/kg day, i.p.) before lethal radiation (750 rad). Survival of mice was compared to a placebo treated group (5 mice) using Kaplan-Meyer statistics (Prizm software). It can be seen that a significantly better survival was obtained in Ritonavir treated mice. **19b)** This figure contains an experiment wherein colony forming capacity was evaluated in Ritonavir treated mice. Increase in colony forming capacity in bone marrow derived cells was observed after Ritonavir treatment *in vitro*, as compared to other protease inhibitors (ICE-I = caspase-1 inhibitor, MG132 = reversible proteasome inhibitor). The effect on increase in colony formation extended to all hematopoietic lineages.

Figure 20:

The effects of Ritonavir on the metabolism of HIV transgenic mice. HIV-1 transgenic mice (Tg26, Dickie et al, Virology 185, 109-19, 1991) that lack the HIV protease gene, but display a number of pathologies including malabsorption and kachexia, were treated with the HIV-1 protease inhibitor ritonavir (10 mg/kg daily i.p. injections) to assess the effects of the drug on a "stressed" metabolism. A group of ten PI-treated mice was compared in their weight changes to a control group of nine animals and the percentage of change was calculated. After 42 days of treatment a statistical significant difference between the total body weight in the two groups had developed ($p < 0.2$).

Figure 21:

The effects of Ritonavir on IL-8 production. Reduction of IL-8 production in HUVEC (human vascular endothelial cells) after treatment with Ritonavir. Activated, proliferation human vascular endothelial cell (HUVEC) cultures were treated with the PI ritonavir (30 nanomol/l) and, after two days, supernatants were collected for ELISA analyses of IL-8 production. Comparison with IL-8 levels of

untreated cultures indicate a decrease in levels of this cytokine by the PI-treatment. This data supports the discovery that protease inhibitors have anti-inflammatory effects that influence endothelial cell activation and proliferation, thus inhibiting mechanisms that also can lead to tumor neovascularization.

Figure 22:

The effects of Ritonavir on NFAT activiation. NFAT activation by HHV8 orf-74 is inhibited by Ritonavir. The nuclear factor of activation in T cells (NFAT) represents an important element in the regulation of cellular functions. Viral genes, such as the human herpes virus 8 (HHV-8) derived open reading frame 74(orf-74) are known to activate NFAT in order to gain control over infected or bystander cells so as to promote viral replication and spread. A luciferase reporter gene assay, where the luciferase expression is regulated by NFAT binding to a promotor element, was used in Jurkat T cells to demonstrate the inhibitory effect of protease inhibitors on NFAT activation. Protein lysates of orf-74 co-transfected cells were prepared from cultures after 12 hours of treatments as indicated in the figure, and luciferase activity was measured by luminescence. This represents yet another example for the regulation of cellular pathways by protease inhibitors that were designed to target a viral protease.

Figure 23:

Inhibition of HIV-Tat induced NFkB activity by Ritonavir. Ritonavir inhibits NFkB activity induced by HIV-1 Tat protein. The nuclear factor kappa B (NFkB) translocates from the cytoplasmic compartment of a cell to the nucleus, where it binds to promotor elements of cytokine- and other regulatory genes, thus initiating changes in cellular activation and function. The HIV-encoded Tat protein is known to induce the activation of the cellular NFkB-pathway. Luciferase-reporter gene constructs that are responsive to NFkB activation were used to measure the effect of cotransfected HIV-1 tat-gene products in Cos cells on NFkB under the influence of PI-treatment. As depicted in the figure, luciferase activity in protein

extracts from PI treated cells was inhibited in a dose-dependent fashion, indicating that ritonavir directly affects cellular pathways and functions.

Figure 24:

Long-term engraftment in Ritonavir-treated mice. Flow scattergram, representative example of each group of mice: upper left panel - not transplanted, lower left panel - transplanted without treatment, upper right panel - transplanted with Ritonavir treatment, lower right panel - transplanted with IFN-gamma treatment.

Figures 25a and 25b:

Inhibition of tumor formation and growth. Kaposi sarcoma derived tumor cells (KSIMM) were injected (7x10e6 per mouse) subcutaneous into immune deficient Beige Nude XID mice. Six days post KSIMM injection, when visible tumors were observed, treatment with Ritonavir was initiated (30 mg ritonavir/kg, intra peritoneal). The control group of animals was injected with Phosphate Buffered Saline (PBS, placebo treatment). After 2 weeks of treatment, a significant inhibition of tumor growth was observed in animals treated with ritonavir. Representative examples of the treatment groups (6 animals in each group) are depicted in the fotographs. **B)** Kaposi sarcoma derived tumor cells (KS-Y1) were injected (5x10e6 per mouse) subcutaneous into immune deficient Beige Nude XID mice. Five days post KS-Y1 injection, when visible tumors were observed, treatment with Ritonavir was initiated (30 mg ritonavir/kg, intra peritoneal). The control group of animals was injected with Phosphate Buffered Saline (PBS, placebo treatment). Tumor size was measured and volumes calculated as illustrated (means+SD are calculated from 5 animals per group)

Figures 26a and 26b:

Inhibition of tumor growth in BNX mice by Ritonavir Control group -placebo- consisted of four animals, Ritonavir treated group = 5 animals; tumor size illustrated in mm^2). Treatment was started after 10 days. **B)** Chronic Myelogenous

Leukemia derived cells (line K562, human) were injected s.c. into immune compromised BNX mice (1.5x10e6 cells per mouse) and after development of visible tumors (at day 5) treatment was initiated with daily i.p. injections of 30mg/kg ritonavir (labelled "treated") or PBS (untreated = placebo group). Tumor growth was measured and volumes were calculated as depicted (means±SD of four mice in ritonavir group, five mice in the placebo group).

Figures 27a and 27b:

Inhibition of tumor growth in Balb/C mice by Ritonavir. Monocytoid tumor cell growth (line U937, human) in immune competent mice (Balb\c strain) is inhibited by ritonavir treatment. Cells ($2x10^7$ per mouse) were injected s.c. and treatment (i.p. injections as described above) was initiated one day post tumor cell injection. In the placebo group (PBS), progressive tumor growth was noted as early as on day four (5 mice per group), whereas a visible tumor developed only in one mouse (out of five) within the ritonavir treated group on day 8. This tumor disappeared after day 16 post inoculation. **B)** Malignant hematopoietic progenitor cells (line KG1a, human, 1x10e7) were injected s.c. into Balb\c mice. Treatments (as described above, 6 animals per group) were initiated at the same time. Development of subcutaneous tumors was monitored over 15 days at which time the experiment was terminated. Mean values are plotted, SD≤18%. Note: inhibition of tumor formation takes place in immune deficient as well as in in immune competent animals, indicating that anti tumor effects are not dependent upon tumor CTL response or closely related anti-tumor mechanisms.

Figure 28:

Inhibition of cell adhesion by Ritonavir

This figure contains the results of an experiment that measured the inhibition of cellular adhesion as a functional readout of Ritonavir and MG132 (proteasome inhibitor) mediated inhibition of cellular activation (relevant for inflammation, lymphocyte infiltration and neo-vascularization). Indicator cells (HL-60 and

U937) were loaded with intracellular fluorescent dye (Molecular Probes, Inc., calcein AM) and after application of defined sheer forces, the amount of adhering cells was measured by fluorescence intensity per culture (WALLACE systems).

Figure 29:

Restoration of hematopoietic function by Rritonavir. Hematopoietic functions are restored by ritonavir treatment in TG26 mice after 3 weeks of treatment as assessed in (hemato-poietic progenitor cell) colony formation assays (CFU-assay) using standard methylcellulose cultures (StemCell Technologies). HIV transgene expression as measured in RNA extracts from tail clippings, that were probed with full length HIV-1 probes (Northern blot) is decreased by ritonavir treatment.

DETAILED DESCRIPTION OF THE INVENTION

Prior to describing the preferred embodiments, the following definitions are provided.

Molecules or compounds of the invention - The molecules or compounds of the invention are molecules or compounds that target, bind to, or interact with a protease and thereby modulate at least one of its activities. Preferably, such molecules or compounds will comprise a protease inhibitor, preferably a microbial or viral protease inhibitor, and more preferably HIV-1 protease, proteasome, serine protease, or cysteine protease inhibitor. Examples thereof include, e.g., Ritonavir, Saquinavir, Nelfinavir and Indinavir, MG132, lactacystin, or cytochrome P450 inhibitor (terminal oxidase of the cellular microsomal mixed-function monoclonal-oxygenase system).

HIV-1 Protease inhibitor - A class of molecules of the invention that inhibit function of HIV-1 function.

HIV-1 Protease - A protease produced by HIV that functions to specifically cleave the HIV-1 Gag-Pol polypeptide into Gag and Pol proteins, which cleavage is essential for viral assembly and maturation of HIV-1.

Proteasome - A multi-subunit complex that degrades specifically targeted proteins which is found in all eukaryotic cells and some bacteria. This protease complex is responsible for processing peptides for presentation on major histocompability complex 1 (MHC-1) molecules on the surface of cells.

Serine Protease - A protease that cleaves a peptide or protein at a site that comprises a serine residue.

Cysteine Protease - A protease that cleaves a peptide or protein at a site that comprises a cysteine residue.

Apoptosis - Programmed cell death.

Host- A host includes humans, non-human primates, non-human mammals, and ungulates. Especially included are agricultural animals, and domestic animals, such as dogs and cats.

The present invention is broadly directed toward the use of molecules that target proteases, especially microbial or viral proteases, or proteasome to modulate cellular pathways, e.g., pathways involved in cell metabolism, cell activation, cell proliferation, cell differentiation, cell maturation, cell cycle and cell death, or to induce systemic changes in metabolism such as changes in sugars, lipids, or protein metabolism.

In particular, such modulation of cellular pathways or systemic metabolism using a protease inhibitor will be utilized to effect human or animal therapy, e.g., in agricultural, veterinary, or therapeutic applications.

More specifically, the present invention is directed toward the use of microbial or viral protease inhibitors or HIV-1 protease, proteasome, serine protease, or cysteine protease inhibitors in particular to treat diseases and conditions including cancer, autoimmune disorders, keratinization disorders, infection (including non-HIV infection), transplantation, graft-vs-host or host-vs-graft disease, as an adjunct to radiation, immuno- or chemotherapy, acute ischemia, allergic disorders, inflammatory disorders such as arthritis, psoriasis,

chronic inflammation, inflammatory bowel syndrome, conditions associated with aging, wasting syndrome, disorders associated with abnormal metabolism, such as those that affect sugar, lipid and protein metabolism.

Although the preferred embodiments of the present invention as described below are in the context of HIV protease inhibitors or proteasome inhibitors, one skilled in the art will recognize that other molecules of the invention can be used without altering the scope of the invention, i.e., inhibitors of other proteases, or proteasome inhibitors. In particular, serine protease inhibitors and cysteine protease inhibitors are intended to fall within the scope of the present invention. Therefore, in all instances HIV-1 protease inhibitor should be construed to be exemplary of protease inhibitors which are suitable for use in the claimed methods. In preferred embodiments, the protease inhibitor will be a molecule that inhibits cellular proteasome or HIV-1 protease.

HIV-1 proteases function to specifically cleave the HIV-1 Gag-Pol polypeptide into Gag and Pol proteins. This post-translational cleavage step is essential for viral assembly and maturation of the virion (Fields et. al., *Fields Virology*, 3rd ed., Lippincott-Raven Publishers, Philadelphia, 1996). The crystal structure and binding site specificity of the HIV-1 protease have been defined, allowing for the design of protease inhibitors which interfere with substrate binding and protease function. In addition to inhibiting HIV proteases, it has also been shown that these inhibitors also inhibit the functions of cellular proteasome, a multi-subunit complex of proteases that degrades specifically targeted proteins. Proteasome, which are found in all eukaryotic cells and in some bacteria such as archaebacteria, are responsible for producing peptides for presentation on major histocompatibility complex I (MHC-I) molecules on the surface of cells (Janeway et. al., *Immunobiology*: The immune system in health and disease, 2nd ed., Garland Publishing, New York, 1996). Thus, inhibitors of proteasome prevent antigen

presentation on MHC class I molecules resulting in a partial loss of immune function.

HIV protease inhibitors and proteasome inhibitors are well known in the art. Examples of such inhibitors include, but are not limited to, the following compounds including derivatives or analogs thereof: HIV protease inhibitors- Saquinavir (Invirase and Fortovase), Ritonavir (Norvir), Indinavir (Crixivan) (Lacy et. al., *Conn Med*, 60(12): 723-7, 1996), Nelfinavir (Viracept) (Kaldor et. al., *J Med Chem,* 40(24):3979-85, 1997), Amprenavir (Agenerase) (No Authors Listed, *Med. Lett. Drugs. Ther*, 41 (1057): 64-66, 1999), Palinavir (Lamarre et. al., *Antimicrob Agents Chemother*, 41(5):965-71, 1997), nonpeptidic HIV protease inhibitors (Vara Prasad et. al., *Bioorg. Med. Chem. Lett.*, 9 (11): 1481-6, 1999), for example, Tipranavir (PNU-140690) (Poppe et. al., *Antimicrob Agents Chemother*, 41(5):1058-63, 1997), small dipeptide-based HIV protease inhibitors, HIV protease inhibitors containing allophenylnorstatine (an unnatural amino acid), piperazine hydroxyethylamine based HIV protease inhibitors, hydroxyethylamine based HIV protease inhibitors, tetrahydropyrimidinone containing HIV protease inhibitors, cyclic HIV protease inhibitors such as cycloalkylpyranones and cycloalkyldihydropyrones (Romines et. al., *J Med Chem*, 39(20): 4125-30, 1996), cyclic urea-based HIV protease inhibitors such as DMP 323 (Erickson-Viitanen et. al., *Antimicrob Agents Chemother*, 38(7):1628-34, 1994), HIV protease inhibitors containing beta-hydroxyether and thioether dipeptide isostrene surrogates (Patel et. al., *Bioorg. Med. Chem. Lett.* 8(8): 931-4, 1998), KNI-577 (Kiso et. al., *Arch. Pharm.*, 331(3): 87-9, 1998), Pyrone based HIV protease inhibitors such as PNU-103017 (Zhong et. al., *Chirality*, 10(3): 210-6, 1998), SC-52151 (Bryant et. al., *Antimicrob Agents Chemother*, 39(10):2229-34, 1995), water soluble HIV protease inhibitors such as R-87366 (Komai et. al., *Biol. Pharm. Bull.*,20(2): 175-80, 1997), hydroxyethylene based derivatives (Lazdins et. al., *Schweiz Med Wochenschr*, 126(43):1849-51, 1996), acetyl pepstatin, tripeptide

based protease inhibitors such as KNI-272 and KNI-102 (Kiriyama et. al., *Biopharm Drug Dispos*, 17(9): 739-51, 1996 and Mimoto et. al., *Chem Pharm Bull* 39(11):3088-90, 1991, respectively), aminodiol based HIV protease inhibitors (Chen et. al., J Med Chem, 39(10): 1991-2007, 1996), BILA 2185 BS (Liard F, et al. *J Pharm Biomed Anal*, 14(1-2):151-4, 1995), hydroxyaminopentane amides such as L-735,524 (Vacca et. al., *Proc Natl Acad Sci*, 91(9):4096-100, 1994), HOE/BAY 793, CGP 53,437 (Flesch G, et al. *J Chromatogr B Biomed Appl*, 657(1):155-61, 1994), non-peptidyl based HIV protease inhibitors (Otto et. al., *Antimicrob Agents Chemother*, 37(12):2606-11, 1993), C2 symmetry-based HIV protease inhibitors such as A77003 (Kageyama et. al., *AIDS Res Hum Retroviruses*, 10(6):735-43, 1994), KNI-102, L-682,679 (deSolms et. al., *J Med Chem* 34(9):2852-7, 1991), and L-687,908 (Vacca et. al., *J Med Chem* 34(3):1225-8, 1991); Proteasome inhibitors-PI31 (Zaiss et. al., *FEBS Lett*, 457(3): 333-8, 1999), epoxomicin (Meng et. al., *Proc Natl Acad Sci*, 96(18): 10403-8, 1999), PS-341 (Adams et. al., *Cancer Res*, 59(11): 2615-22, 1999), lactacystin and derivatives thereof, CVT-634 (Lum et. al., *Biochem Pharmacol*, 55(9): 1391-7, 1998), dipeptidyl based proteasome inhibitors such as CEP1612 (An et. al., *Cell Death Differ*, 5(12): 1062-75, 1998), AcLLnal, peptidyl aldehyde based inhibitors such as Z-LLF-CHO (Orlowski et. al., *Cancer Res*, 58(19): 4342-8, 1998), CF-2 (Guo et. al., *J Biol Chem*, 269(17): 12399-402, 1994), and alpha-ketoamide based proteasome inhibitors (Joly et. al., WO/ 9937666).

In the search for methods to enhance immune responses, the inventors have observed the surprising and unexpected phenomenon that HIV-1 protease inhibitors (PR-I) and proteasome inhibitors are potent immune modulators. The ability of PR-I to modulate the immune system is independent of HIV-1 protease inhibition and is not related to HIV infection, since similar effects were observed in HIV free experimental systems. The inventors have found that exposure of cells (human and murine) to HIV-protease inhibitors, more specifically Ritonavir

(Norvir), at concentrations that are achieved in humans under therapy for HIV disease effect changes in cell activation, proliferative capacity, and function. Further, these changes include but are not limited to an enhanced ability of cells to survive differently induced stress conditions (e.g., metabolic, apoptotic and the like).

Based on experimental results, some of which are discussed in the Examples *infra*, it is hypothesized that protease inhibitors may elicit effects on cellular pathways that affect immune and other cellular reactions.

First, it has been observed that protease inhibitors such as HIV-1 protease or proteasome inhibitors affect cytokine expression, i.e., the specific cytokines which are expressed and the levels of expression thereof are cytokines well known to have profound effects on immunity, e.g., they are involved in inflammatory responses, and whether a host elicits a Th1 (cellular) or Th2 (humoral) immune response against a particular antigen. Based on such effects, protease inhibitor administration affords a means of modulating cytokine expression, and thereby the various cellular and immune effects that are affected thereby. For example, it may provide for the activation of different types of T-cells.

Secondly, it is theorized that protease inhibitors such as proteasome inhibitors may interact with chaperones such as Heat Shock Protein (HSP70, HSP72) resulting in the activation of expression thereof. This in turn is believed to result in an increase in the amount of antigen that is processed and presented by antigen-presenting cells. Such increase results in enhanced immune responses. Still further, it appears, based on the results discussed *infra*, that protease inhibitors modulate apoptosis and neovascularization.

Thus, based on such observations, the present invention is directed toward the use of a protease inhibitor such as an HIV-1 protease or proteasome inhibitor to modulate cellular pathways, in particular those that regulate cell metabolism, cell activation, cell proliferation, cell differentiation, cell mutation, cell cycle and

cell death. Accordingly, the invention will be used to treat or prevent any disease or condition wherein modulation of any of the foregoing cellular pathways provides an effective means of treatment or prophylaxis.

In particular, the present invention is directed toward the use of protease inhibitors to treat disorders or conditions involving abnormal cell metabolism such as cancer or dermatological conditions.

Also, the present invention is directed toward the use of a protease inhibitor to treat disorders or conditions involving aberrant cell activation, proliferation or differentiation. Such conditions include, by way of example, autoimmune disorders, inflammatory conditions, cancers, keratinization disorders, dermatological disorders or autoimmune diseases.

Further, the present invention is directed toward treatment of diseases and conditions wherein modulation of cell cycle or cell death is desirable. Such conditions include, by way of example, cancer, infection, metastasis, aging or wasting syndrome.

In accordance with one embodiment of the invention, there is provided a method to modulate the immune system of a host comprising administering a therapeutically effective amount of a molecule or compound of the invention such as an HIV protease inhibitor or proteasome inhibitor.

For example, an HIV protease inhibitor can be used to modulate one aspect of the immune system, immune cell activation. Essentially, an effective amount of the protease inhibitor is administered to a patient with a condition or disease of the immune system in order to modulate immune cell activation. The dose of protease inhibitor to be administered can be determined by methods well known in the art. Such conditions including, by way of example, inflammatory diseases, autoimmune disorder, allergic disorder, transplant, graft-vs-host disease, host-vs-graft disease.

The protease inhibitors of the invention may be administered to a human or other animal in an amount sufficient to produce a therapeutic, prophylactic, cosmetic or dermatological effect. Suitable protease inhibitors of the invention, e.g., HIV protease inhibitor, or proteasome inhibitor, can be administered to such human or other animal in a conventional dosage form prepared by combining an HIV protease inhibitor or proteasome inhibitor, or other protease inhibitor, with a conventional pharmaceutically, cosmetically or dermatologically acceptable carrier or diluent according to known techniques. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables.

The route of administration of the protease inhibitor (e.g., HIV protease inhibitor or a fragment thereof) of the invention may be oral, parenteral, by inhalation or topical. The term parenteral as used herein includes intravenous, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration. Subcutaneous and intramuscular forms of parenteral administration are generally preferred. However, the preferred mode of administration will vary, e.g., dependent upon the particular condition treated.

The daily parenteral and oral dosage regimens for administering protease inhibitor according to the invention will generally be at concentrations that are achieved in humans under therapy for HIV disease. In a preferred embodiment, the daily parenteral and oral dosage regimens for employing compounds of the invention will be in the range of about 0.05 to 100, but preferably about 0.5 to 20, milligrams per kilogram body weight per day.

The protease inhibitors of the invention, e.g., HIV-1 protease inhibitor or proteasome inhibitor, may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may

be prepared by conventional techniques. The preferred dosage amount of a compound of the invention to be employed is generally within the range of about 10 to 100 milligrams.

The protease inhibitors of the invention, e.g., HIV-1 protease inhibitors or proteasome inhibitors, may also be administered topically. By topical administration is meant non-systemic administration and includes the application of an HIV protease inhibitor (or a fragment thereof) of the invention externally to the epidermis, to the buccal cavity, a instillation of such an HIV protease inhibitor into the ear, eye and nose, where it does not significantly enter the blood stream. This mode of administration is particularly desirable in the context of cosmetic or dermatological applications of the invention, e.g., in skin creams, ointments, or other topically administrable forms for the treatment of the skin. By systemic administration is meant oral, intravenous, intraperitoneal and intramuscular administration. The amount of a protease inhibitor, e.g., HIV-1 protease inhibitor, required for therapeutic or prophylactic effect will, of course, vary with the particular protease inhibitor chosen, the nature and severity of the disease or condition of the immune system being treated and the human or animal undergoing treatment, and is ultimately at the discretion of the physician. A suitable topical dose of an HIV protease inhibitor or proteasome inhibitor will generally be within the range of about 1 to 100 milligrams per kilogram body weight daily. In a preferred embodiment of the present invention, the protease inhibitor is a type one HIV protease inhibitor, such as Ritonavir (Norvir).

In another embodiment of the invention, protease inhibitors such as HIV-1 protease or proteasome inhibitors, are administered to effect changes in immune cell proliferative capacity and cell function. For example, an HIV protease inhibitor can be administered to a patient to increase or decrease immune cell proliferation of particular types of immune cells, e.g., particular T-cell subsets.

Yet another embodiment of the invention involves modulating non-immune cell proliferation. This can be effected *in vivo* or *in vitro*, e.g., an inhibitor can be used to treat bone marrow during purging procedures, e.g., effected during treatment of B-cell lymphoma. For example, a protease inhibitors can be used to support cell generation poiesis, including blood cell growth and generation (pro-hematopoietic effect) after depletion or destruction of cells, as caused by, for example, toxic agents, radiation, immunotherapy, growth defects, malnutrition, immune dysregulation, anemia and the like. Further, the protease inhibitors of the invention can provide a therapeutic control of tissue generation and degradation, and a therapeutic modification of cell and tissue maintenance and blood cell homeostasis.

In HIV infected patients, the therapeutic application of HIV protease inhibitors commonly leads to a substantial increases in CD4+ T-cell counts but, intriguingly, not always to an immediate decrease, or to only insignificant decreases, in HIV viremia (Levitz et al., *New England J. Med.*, 338/15, 1074, 1998). Even after the initial decrease of plasma HIV load, a viral "break-through" is frequently observed while T-cell counts remain stable and increased (as compared to the time of therapy-initiation). Thus, HIV-PR-I and other protease inhibitors may have additional beneficial effects on T-cell homeostasis.

Thus, another use of HIV-1 and other protease inhibitors is for the treatment of disorders involving T-cells such as T-cell related cancers, T-cell related autoimmune disorders, inflammatory conditions, infections, aplastic anemia, multiple sclerosis, and DiGeorge syndrome.

Further, marked changes in T-cell subset distribution and lymphocyte function have been observed including increases in "memory" CD4CD45RO+ cells and in "naïve" CD4CD45RA+ cells after initiation of HIV protease inhibitor therapy. Reduced expression of activation markers on immune cells such as CD38, CD69 and CD95 as well as restored *in vitro* proliferative responses to

mitogens and recall antigens correlated with the clinical improvement of patients that are compliant with PR-I treatment regimens (Kelleher et al., *Journal of Infectious Diseases*, 173:321-329, 1996). The time course of these changes (when they occur and when they become detectable) depends on the methods and specific criteria, as well as the kind of HIV PR-I used. The overall finding of the invention hinges on the discovery that HIV protease inhibitors, although specifically designed to block HIV protease activity, have additional and direct effects on immune cell functions. These "side effects" may represent an important part of HIV PR-I's therapeutic effects. Thus, it is yet another embodiment of the invention to use protease inhibitors, and HIV protease inhibitors or proteasome inhibitors in particular, to modulate T-cell subsets and lymphocyte function.

Using *in vitro* and *in vivo* systems (HIV-free cell culture systems and animal models), the inventors have also discovered that the HIV protease and proteasome inhibitors Ritonavir, for example, applied at therapeutic or lower concentrations, increased the colony forming capacity (CFC) of human bone marrow derived mononuclear cells (BM-MNC). In addition, peripheral blood derived T-cells responded with phenotypic changes in cell surface marker expression including CD25, CD28, and CD95(Fas)-ligand, as well as with changes in susceptibility to CD95 mediated apoptosis. Further, treatment of BALB\c mice with HIV-PR-I for three weeks led to a significant increase in peripheral blood white blood cell counts. These results indicate that HIV protease inhibitors have immune enhancing properties. Thus, it is another embodiment of the invention to use a protease inhibitor, or HIV protease inhibitor or protease inhibitor, specifically, to enhance the immune system.

Based on the above-observed effects of HIV protease inhibitors which are described in the Examples which follow a preferred embodiment the invention is directed toward the use of PR-I treatment for modulating and/or enhancing the immune system and, more specifically, the cellular arm of the immune system in

a subject in need of such modulation and/or enhancement. Suitable PR-Is include HIV protease inhibitors, proteasome inhibitors, serine protease inhibitors and cysteine protease inhibitors. Boosting of the immune system may be beneficial in the treatment of other disorders wherein the host's immune system is compromised or overburdened, e.g., infection, shock, trauma, starvation and metabolic disorders such as malabsorption.

In particular, PR-I administration can be used during cancer treatment and/or prophylaxis. It is anticipated that PR-I administration to cancer patients, or persons at increased risk of developing cancer, e.g., because of genetic reasons and/or age, will be administered at least one PR-I in order to boost and/or modulate the immune system, thereby resulting in effective treatment and/or prophylaxis of cancers. It is anticipated that the administration of one or more PR-Is will enhance cellular immune processes and immune surveillance, thereby potentially providing for the eradication and/or reduced number of malignant cells. Also, the inhibitor may elicit an anti-cancer effect by inducing apoptosis, inhibition of neovascularization and by increasing host immune responses against cancer cells. Further, as discussed previously, it is hypothesized that proteasome inhibitor may in conjunction with chaperones, e.g., heat shock proteins such as hsp70, triggers an increase in antigen processing and presentation by antigen presenting cells.

Such protease inhibitor can be used by itself or in conjunction with other anti-cancer treatments or prophylaxis, e.g., chemotherapeutics, radiation, other immune modulators, cytokines, and immunotherapeutics. Cancers which should be treatable and/or preventable according to the invention include, by way of example, breast, prostate, liver, bladder, lung, esophageal, stomach, skin, pancreatic, brain, uterine, colon, brain, head and neck, and ovarian cancer. Of course, in its preferred embodiment, PR-I treatment will be effected in the early

stages of cancer, i.e., at a point wherein enhanced immune surveillance can potentially provide a cure.

Another significant aspect of the invention is in the area of vaccines, both prophylactic and therapeutic vaccines. It is anticipated that PR-I treatment before, proximate, or after the administration of a vaccine composition will potentiate or alter the immune response of a particular subject, i.e., the cellular arm, for example by enhancing or resulting in a CTL response, thereby enhancing the efficacy of the vaccine composition. Protease inhibitors may act to shift the immune response, e.g., from a Th_2 to a Th_1 response, thereby enhancing cellular immune functions. This is particularly desirable in the context of vaccines against infectious agents such as viruses, bacteria, parasites such as plasmodia, trypanosomes, and fungi, among others. PR-I treatment should shorten the necessary time for the host to amount an appropriate immunological response, i.e., "defense", against the particular agent to which the vaccine is specific. In addition, PR-I treatment should broaden the immune response of a vaccine. This should reduce the risk of chronic adverse responses such as inflammatory or infectious responses. Thus, PR-1 treatment will be beneficial in treating any condition wherein mounting or maintaining a Th_1 response is therapeutically desirable.

Infectious conditions wherein PR-I treatment should be beneficial include, by way of example, Lyme disease, herpes, hepatitis, parasite infection such as plasmodia, trypanosomes, schistosomiasis, and other chronic infections such as those induced by other viruses, bacteria and fungi. PR-I administration may be combined with vaccine administration or may be effected separately.

Another embodiment of the invention comprises the administration of a PR-I for the treatment and/or prophylaxis of inflammatory diseases and conditions, e.g., psoriasis, arthritis, inflammatory bowel syndrome, chronic inflammation and chronic diarrhea.

Another embodiment of the invention comprises the administration of a PR-I to bolster the immune system of a patient prior to surgery. PR-I administration and the resulting immune/nonimmune cell proliferation would avoid the need for transfusions and limit post surgical risks of infection.

Yet another embodiment of the invention involves the treatment of persons who are immuno-compromised, for example because of disease or trauma. PR-I treatment will be beneficial in that it should alleviate symptoms of the disease or trauma associated with an impaired immune response, and also avert complications which may arise as a result of immuno-compromised states, such as post-operative infection, post-trauma infection, cancer, or other conditions which may occur in persons that are immuno-compromised. Examples of persons who are immuno-compromised wherein PR-I administration should be beneficial include transplant recipients, persons receiving immunosuppressants, persons immuno-compromised because of genetic reasons, persons who have experienced shock or trauma, burn victims, and persons undergoing radiation or chemotherapy, or any treatment that results in an immuno-compromised state. The administration of one or more PR-I should potentiate or modulate the immune system and mechanisms associated therewith, thereby averting the risk of infection or other immunologically related diseases and, moreover, should also potentiate the body's natural repair processes. It is hypothesized, based on the results observed by the present inventors, that this enhances the immune response, e.g., by altering cytokine expression, which act to drive the immune response or by affecting the interaction of proteasome with chaperones, e.g., hsp70, the expression of which triggers an enhance in antigen presentation by APCs.

Thus, PR-I treatment in the context of the invention can be broadly construed to be an immune "adjuvant" which acts to boost or enhance the immune system of subjects in need of such treatment. Other examples of conditions wherein immune suppression is a problem which may be treated according to the

invention include retroviral infections, such as HIV-1 and HIV-2, cancer, papilloma viridae, herpes viridae, HTLV-I and -II.

As discussed above, PR-I treatment may be used to effect specific types of immune processes, e.g., T-help type I responses (Th1) that may result in a specific cytotoxic lymphocyte response. This is in contrast to Th-2 responses which result in enhancement of humoral immune responses (enhanced antibody response). The results obtained by the inventors suggest that PR-I administration may actually alter the type of immune response that is elicited against a particular antigen. Based on such activity of protease inhibitor treatment, PR administration may actually change the means by which a vaccine or antigen composition affects the immune system. For example, administration of a protease inhibitor may result in the immune system eliciting a cellular (e.g., CTL) response against a pathogen rather than a humoral immune response. This may be particularly beneficial in the context of diseases wherein humoral immune responses are thought to be of little benefit or perhaps even detrimental to the host's defense against a particular disease or infectious agent, e.g., parasite infection or anti-tumor immunity.

Also, protease inhibitor treatment, because of its effect on cell proliferation, especially cell generation and poiesis, can be used in the treatment of persons wherein increased cell proliferation and poiesis is beneficial. Examples thereof include subjects undergoing radiation or chemotherapy, persons with growth defects, shock and trauma victims, persons with other injuries, malnutrition, persons who have suffered significant blood loss, persons who have received a tissue graft, anemia, persons suffering from immune dysregulation, and other conditions or persons wherein increased cell proliferation is therapeutically desirable. PR-I treatment should also be useful for maintaining tissue regeneration and promoting tissue degradation, blood cell hemeostasis, and cell and tissue maintenance. Another usage is during wartime, e.g., to persons exposed to

bioharzardous agents, chemicals, mutagens, or radiation. These persons may be in need for treatment because of the adverse effects this may have on the immune system in general, and cell proliferation, hematopoiesis, and metabolism specifically.

Still further, PR-I treatment will be beneficial in protecting cells or tissues from different kinds of death signals (e.g., elicited by toxins, starvation, radiation, hyperactivation, anergy, and apoptosis), for example, the result of trauma, inflammation, infection or transplantation.

Yet another area wherein PR-I treatment should be beneficial is in the geriatric population wherein the immune system, because of age, may be compromised relative to younger persons. PR-I administration may provide for increased immune and other cell proliferation, decreased cell degradation, blood and tissue maintenance, which would otherwise potentially be reduced relative to younger persons. For example, PR-I treatment may be used prophylactically or therapeutically in aged persons in order to enhance or reduce their immune systems, thereby reducing the risk of some cancers, infection, or other conditions and disorders that increase in incidence as a result of age. Also, PR-1 administration may inhibit telomerase activity, which has been reported to play a significant role in aging and cell death.

As noted above, in a preferred embodiment, protease inhibitors are utilized to treat diseases or conditions of the immune system. For example, autoimmune diseases such as lupus, Scleroderma, Sjogren's Syndrome, ITP, diabetes, multiple sclerosis, Graves' disease, IBD, and rheumatoid arthritis can be treated. The HIV protease inhibitors of the invention are also applicable in treating other diseases or conditions which occur or persist due to an impaired or compromised immune system. For example, cancers persist in hosts due to the immune system no longer recognizing antigens presented on the surface of tumor cells. Treatment in accordance with the invention, will enhance anti-cancer immune responses as

mediated by HIV protease inhibitor-activated immune cells resulting in the restoration of immune surveillance.

As discussed previously, another embodiment of the invention comprises the use of protease inhibitors to modulate different aspects of cellular metabolism. For example, cells and tissues can be protected from induced death signals (e.g., by toxins, starvation, radiation, hyperactivation, anergy and apoptosis) produced in situations such as trauma, inflammation, infection and transplantation. In addition, HIV protease inhibitors can be used to alter cellular metabolism in cancer to make cancer cells less neoplastic and more accessible for "conventional" anti-cancer drugs and host cell cycle control. This can be effected *in vitro* or *in vivo*. For example, bone marrow can be treated *in vitro* during purging of bone marrow, e.g., by contacting with a protease inhibitor, to render tumor cells more susceptible to apoptosis. Also, PR-1 administration may be used to treat subjects with wasting syndrome by triggering a shift in cellular metabolism. This is useful in the context of disorders or treatments that may have an adverse consequence on the subject's weight and overall metabolism, such as viral or parasitic infection, cancer, chemotherapy, radiotherapy and gene therapy such as bacterial or viral.

In yet another preferred embodiment of the invention, protease inhibitors, such as HIV-1 protease or proteasome inhibitors, are utilized to modulate or decrease anti-rejection responses in hosts receiving transplants and/or grafts. For example, patients who have undergone organ transplantation procedures typically reject the new organ due to the immune system recognizing the organ as "foreign" and attempting to eliminate or destroy it. Administration of a molecule or compound of the invention may inhibit the immune system from recognizing the "foreign" organ, thereby reducing the chance of organ rejection.

As previously discussed, yet another application of protease inhibitors is to modulate apoptosis, i.e., programmed cell death. This is useful in various

therapeutic contexts, e.g., treatment of cancer, or infection, wherein eradication of specific types of cells is therapeutically beneficial.

The pathophysiological role of apoptosis in disrupting homeostasis in, for example, HIV infections has been well established (Thompson CB: Apoptosis in the pathogenesis and treatment of disease. *Science*, 267:1456, 1995). Several studies have shown that enhanced spontaneous and activation induced apoptosis occur in PBMC and purified T-cells derived from HIV-infected individuals. These processes are thought to be involved in both functional defects and depletion of immune cells that are not infected by HIV (Meyaard et. al., *Science*, 257:217, 1992; Groux et. al., *J. Exp. Med.*, 175:331, 1992). T-lymphocyte apoptosis can be induced by signals mediated by the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family and their respective ligands (Ashkenazi et. al., *Science*, 281:1305, 1998). Others have shown that peripheral blood CD4+ and CD8+ T-cells derived from HIV-infected individuals express abnormally high levels of both death receptors and ligands. In addition, susceptibility of these cells to apoptosis as mediated through T-cell receptor (TCR), CD95, and TNF receptor 1 (TNFR1) signals is increased, and can be further enhanced by interferon (IFN)-alpha and IFN-gamma (Sloand et. al., *Blood*, 89:1357, 1997; Sloand et. al., *J. Clin. Invest.*, 101:195, 1998; Debatin et. al., *Blood*, 83:3101, 1994; Oyaizu et. al., *Blood*, 82:3392, 1993; Katsikis et. al., *J. Exp. Med.*, 181:2029, 1995).

Clinical data indicate that both the apoptosis receptor expression and the percentage of apoptotic cells declines most significantly during anti-retroviral therapy that includes HIV-PR-I (Johnson et. al., *Clin. Exp. Immunol.*, 113:229, 1998; Lederman et. al., *J. Infect. Dis.*, 178:70, 1998; Badley et. al., *J. Clin. Invest.*, 102:79, 1998; Bohler et. al., *Blood*, 90:886, 1997; Flexner C: HIV-protease inhibitors. *New England J. Med.*, 338:1281, 1998). Thus, it is yet another embodiment of the invention to utilize protease inhibitors, and HIV protease

inhibitors or proteasome inhibitors specifically, to modulate apoptosis, e.g., for the treatment of infection, cancer, metabolic disorders, malnutrition and mal-absorption.

Also, yet another application of HIV-1 protease or proteasome inhibitors is for the treatment of HIV-2 infection. The efficacy of such treatment was shown in monkeys and clearly is a function of the immune stimulating properties of the protease inhibitors as HIV-2 is not cleaved by HIV-1 protease.

The foregoing is exemplary and not exhaustive of the myriad of applications wherein PR-I administration will be beneficial. Conceivably, PR-I administration can be used as an adjunct or treatment of any condition wherein the immune system is desirably boosted, enhanced, or altered.

Having described the preferred embodiments of the present invention, one skilled in the art will recognize that modifications can be made to the preferred embodiments without altering the scope of the invention.

The following examples are provided to further describe the invention, however, the scope of the invention is not limited thereby.

EXAMPLE I

Cell Separation and Culture

Cell separation and culture were performed using standard technique as described (Sloand et. al., *Blood*, 89:1357-1363, 1997; Sloand et. al., *J. Clin. Invest.*, 101/1, 1-7, 1998).

Cell Separation

Peripheral blood samples were obtained from healthy volunteers according to the protocols approved by the Institutional Review Board. PBMCs were isolated by automated Ficoll/Hypaque density-gradient centrifugation using a CS-3000 Plus Blood Cell Separator (Fenwal Division, Baxter Healthcare Corp., Deerfield, IL). Monocytes were isolated from PBMCs using counterflow-centrifugal elutriation utilizing a Beckman JE-5.0 rotor and a type A

chamber (Beckman Instruments, Inc., Palo Alto, CA). Purity of the separated monocyte fraction was approximately 95% as determined by cytomorphology in Pappenheim stain and approximately 96% as determined by the expression of CD14 antigen (LeuM3; Becton Dickinson, Mountain View, CA) and measured by flow cytometry.

Cell Culture and supplements

T-cells were cultured in complete medium (RPMI 1640, containing 10% heat inactivated FCS, low endotoxin grade; all GIBCO BRL, Gaithersburg, MD) at an initial concentration of $0.5x10^6$ cells/ml. PBMC culture media was supplemented with IL-2 (20 U/ml, Boehringer Mannheim, Indianapolis, MN), 0.1 mg/ml penicillin G and 0.25 mg/ml streptomycin (both Gibco-BRL). For toxicity evaluations, Ritonavir (Abbott Laboratories, North Chicago, IL) was added to 15-day-cultures to obtain different final concentrations ranging from 0.1 nmol/l to 10 μ mol/l. Medium and drug were replaced daily by exchanging 75% of the culture supernatants, and every four days the cultures were split equally as necessary to prevent overgrowth.

IFN-alpha (300 IU/ml, recombinant IFN-alpha-2b, Schering Corporation, Kenilworth, NJ), IFN-gamma (30 ng/ml, Biosource, Camarillo, CA), and TNF (TNF-alpha, 10 ng/ml, BioSource) were added to the cultures 28 hours post activation with anti CD3 mAb (#30110D, 0.1 μg/ml, PharMingen, San Diego, CA) and maintained for three days. Aliquots of differently treated cells were collected for cell surface staining and FACS analysis. In Fas-induced apoptosis experiments, anti-CD95 mAb CH11 (Kamyia, San Francisco, CA), an antibody that mimics Fas-L by cross-linking and triggering the CD95 receptor, or mAb ZB4, a CD95 blocking antibody (AMAC, Westbrook, ME), were used at concentrations ranging from 0.01 μg/mL to 0.5 μg/mL.

Elutriated monocytes were cultured in serum free medium (Cellgrow 40-101-LV, Mediatech Inc., Herndon, VA) at a density of $3x10^6$ cells/ml.

Ritonavir (Abbott Laboratories) was added at concentrations ranging from 0.1 nM to 100 nM, before stimulation with lipopolysaccharide (LPS, 10 ng/ml, Sigma Chemicals, St. Louis, MO) or IFN-gamma (50 ng/ml, BioSource).

When appropriate, natural lymphocyte derived interleukin-2 (IL-2, Boehringer Mannheim, Indianapolis, IN), anti-CD3 mAb OKT3 (for AICD experiments) or phytohemagglutinin (PHA, Boehringer) were used for stimulation at concentrations of 10 U/mL, 0.1 μg/ml, or 5 μg/mL, respectively. Anti-Fas mAb CH11 (Kamyia, San Francisco, CA) an antibody which mimics the Fas-L by cross-linking the Fas receptor or ZB4 a blocking anti-Fas mAb (AMAC, Westbrook, ME) was used at 0.25 μg/ml.

EXAMPLE II

Apoptosis Induction and Inhibition Experiments

Freshly prepared PBMCs were cultured for 24 hours at 10^6 cells/ml in RPMI-1640 plus 5% FCS in 24-well plates coated with or without monoclonal anti-Fas antibody CH-11 or anti-CD3 antibody OKT3. Cultures were incubated at 37° C in a 5% CO_2 humid atmosphere. Plates were then coated with 200 μl/well of 5 μ/ml of antibodies in PBS, pH 7.4. After incubating for two hours at 37° C, the plates were briefly washed twice with 500 ml/well RPMI-1640 plus 5% FCS and then washed one additional time as above for 30 minutes at 37° C. In the inhibition experiments, PBMCs were pre-incubated with HIV PR-I, or for comparison, with ICE inhibitors for 16 hours at 37° C in a 5% CO_2 humid atmosphere before being transferred to antibody-coated plates. Different human cell lines were used to identify those that closely resemble the effects of HIV-PR-I on primary human cells. Withdrawal of IL-2 and essential nutrients as well as treatment with IFN-alpha, IFN-gamma and TNF-alpha were used in other assays to induce apoptosis.

EXAMPLE III

Apoptosis and Viability Assays

Apoptosis and viability assays for individual cell subsets have been described in detail (Sloand et. al., *Blood*, 89:1357-1363, 1997; Sloand et. al., *J. Clin. Invest.*, 101/1, 1-7, 1998). In addition, commercially available Apoptosis ELISAs (Boehringer Mannheim) can be used. Cell viability was measured using a standard Trypan blue (Life Technologies) exclusion test or an automated colorimetric assay utilizing tetrazolium bromide (MTT) reduction reaction in which *in situ* reduction of tetrazolium bromide to a blue formazan product by viable cells. The reduction reaction was assayed spectro-photometrically based on a standard curve and correlated to trypan blue exclusion results. The WST-1 reagent can be used for assessment of metabolic activity, according to the manufacturer's instruction (Boehringer Mannheim).

Assessment of DNA fragmentation and Caspase activity

Assessment of DNA fragmentation was performed using the Cell Death Detection ELISA^{plus} according to the manufacturer's recommendations (Boehringer Mannheim GmbH). Caspase-3 (CPP-32) activity in cytosolic preparations was determined using a fluorogenic assay (Cat.# 6632, PharMingen, San Diego, CA), and samples were read in a microplate spectrofluorometer.

EXAMPLE IV

Cytofluorometric Determinations of Apoptosis-associated Alterations in Whole Cells

To evaluate the delta Tm, cells (10^6/ml) were incubated with the cationic lipophilic dye chloromethyl-X-rosamine (CMXRos; 150 nM; Molecular Probes, Inc., Eugene, OR). Delta Tm (transmembrane potential) marks a point of no return for the apoptotic cascade. As a control, cells were simultaneously treated with the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone (mClCCP; 50 μM; Sigma Chemical Co.). CMXRos incorporates into mitochondria driven

by the delta Tm and reacts with thiol residues to form covalent aldehyde-fixable thiol ester bonds. After fixation (4% paraformaldehyde in PBS for 15 min at room temperature), cells were washed and stained for the detection of chromatinolysis using the TUNEL method. In some experiments, cells were stained with the potential-sensitive dye DiOC6(3) (15 min, 37° C, 40 nM) together with a biotin-Annexin V conjugate (50 x dilution; revealed by streptavidine-phycoerythrine at 5 μg/ml, following the manufacturer's protocol; Boehringer Mannheim GmbH, Mannheim, Germany), followed by sorting of DiOC6(3)low Annexin V+, DiOC6 (3) Annexin V and DiOC6(3)high Annexin V+ cells on an Elite cytofluorometer (Coulter Corp., Miami, FL).

EXAMPLE V

Flow Cytometry

For qualitative and quantitative determination of cell surface molecules, flow cytometry was carried out by incubating cells at 4° C for 30 minutes in PBS containing 1% BSA, 5 mM EDTA, and the indicated antibodies at concentrations recommended by the manufacturer. Fluorescein-isothiocyanate (FITC)-labeled or ECD-labeled mAb to CD3 and CD25, phycoerythrin (PE)-conjugated or PE-Cy5-conjugated mAbs to CD4 and CD14, and appropriate isotype control antibodies were obtained from Becton Dickinson (Mountain View, CA). FITC-conjugated anti-CD95 mAb (UB2), and appropriate isotype control antibodies were obtained from PharMingen. Biotinylated anti-CD95-Ligand (Coulter/Immunotech) was developed with Avidin-PE (Becton Dickinson). Cell surface staining for AnnexinV was performed using the Apoptosis Detection Kit from R&D Systems (Minneapolis, MN). Intracellular staining for Casp1 was performed using the Cytofix/Cytoperm Plus™ (PharMingen) permeabilization procedure and anti-ICE (casp1) polyclonal IgG (A-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as previously described (Sloand et. al., *J. Clin. Invest.*, 101:195, 1998).

EXAMPLE VI

Measurements of Cytokines and Chemokines in Supernatants

Levels of cytokines that effect T-cell recruitment, activation and polarization were determined in PBMC and monocyte culture supernatants at different time points post-treatments and stimulation, over a time period of five days, utilizing enzyme linked immunosorbent assay (ELISA) systems (R&D Systems Inc., Minneapolis, MN) following the manufacturers recommendations. Samples were stored at -80°C until assayed.

Measurement of TNF levels

Levels of TNF were determined in PBMC and monocyte culture supernatants at different time points post-stimulation, utilizing an enzyme linked immunosorbent assay (ELISA) system (R&D Systems Inc., Minneapolis, MN) following the manufacturers recommendations. Samples were stored at -80°C until assayed.

EXAMPLE VII

Quantification of mRNA Expression

To measure mRNA expression levels of IFN-alpha, IL-12, IL-6, and TGF-beta, of RANTES, MIP-1alpha and MCP-1, and IL-4, IL-5, IL10 that are of special interest in understanding the immune augmenting effects of proteasome modulation, cells were harvested at different time points and total RNA was isolated using methods well known in the art. The mRNA was quantified using RiboQant^{R} multi-probe RNase protection assay systems (PharMingen) according to the manufacturers' protocol. The ribonuclease protection assay (RPA) is a highly specific and sensitive method for the detection and quantitation of mRNA species. PharMingen developed multi probe RPA systems that generate a series of templates, each of distinct length and representing a sequence in a distinct mRNA species. The templates are assembled into biologically relevant sets. We

have used the following template sets: hCK1-5, hCR5-6, hAPO-1c, hAPO-2, and hAPO-3.

EXAMPLE VIII

Preparation of Cytosols and Determination of the Activity of ICE-like Proteases.

100 μl of cell cytosols (10^7 cells/100 μl in cell-free system [CFS] buffer [220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM PO_4H_2K, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, 1 mM dithiotreitol, 10 mM Hepes-NaOH]) and pH 7.4 buffer (supplemented with additional protease inhibitors: 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml anti-pain, 10 μg/ml chymopapain) were prepared by five freeze/thaw cycles in liquid nitrogen, followed by centrifugation (1.5 x 10^5 g, 4° C, 1 h) as described (Abbas et. al., *Cellular and Molecular Immunology*, W.B. Saunders Company, Philadelphia, (3rd Ed., 1997)). The protein concentration in the supernatant was determined by the Bradford assay (Bio Rad Labs., Hercules, CA). ICE activity was measured using a fluorogenic substrate containing the cleavage site YVAD, 4-(4-dimethylaminophenylazo)benzoic-YVADAPV-5-(-2-aminoethyl-amino)-naphthalene-1-sulfonic acid (Bachem). The capacity of cytosols or purified recombinant human CPP32 activity to cleave the CPP32 recognition site DEVD was determined using Ac-DEVD-amino-4-methylcoumarin (Bachem) as fluorogenic substrate.

EXAMPLE IX

Preparation of Organelles

Mitochondria were isolated from differently treated cells or cell lines that were prepared for the assays. Mitochondria were purified on a Percoll (Pharmacia, Uppsala, Sweden) gradient and stored on ice in CFS buffer (see Example VIII) for up to four hours. Mitochondria were then washed and resuspended in CFS supplemented with 2 mM ATP. Nuclei from HeLa cells were

purified on a sucrose gradient and conserved in 50% glycerol (Sigma Chemical Co.) in HeLa nuclei buffer at 20° C for a maximum of 15 d. Mitochondrial apoptosis inducing factor (AIF) was purified from cells treated with atractyloside (5 mM; Atr; Sigma Chemical Co.) to induce permeability transition (PT) and liberation of apoptosis inducing factor (AIF). Supernatants (150,000 g, 1 hour, 4° C) from these mitochondria were concentrated on Centricon 10 membranes (10 kD; Amicon, Beverly, MA) and then injected into a FPLC column (MonoQ (HR5/5); Pharmacia) pre-equilibrated with protein-free CFS buffer. Elution was performed on a linear gradient from 0 to 250 mM NaCl at 0.5 ml/min over 30 min, followed by elution at 1 M NaCl thereafter. All fractionation steps were carried out at 4° C to avoid loss of biological AIF activity. The active fraction (eluting at 110 mM NaCl) was dialyzed against protein-free CFS buffer (4° C, overnight, 5,000x excess of CFS buffer), concentrated on Centricon 10 membranes, adjusted to a concentration of 30 μg/ml, and aliquoted to be snap frozen in liquid nitrogen and stored at -80° C.

EXAMPLE X

Determination of Mitochondrial PT

For the induction of permeability transition (PT), mitochondria from different cell lines were incubated with cytosolic extracts from Fas-treated and/or HIV PR-I treated cells (standard dose of 30 μg protein/ml), purified recombinant ICE (50 μg/ml), the pro-oxidant ter-butylhydroperoxide (t-BHP; 30 μM), atractyloside (5 mM; Sigma Chemical Co.), the protonophore mClCCP (100 μl; Sigma Chemical Co.), bongkrekic acid (50 μM), monochlorobiman (30 μM; Sigma Chemical Co.), and/or the calpain inhibitor N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-tyrosine diazomethylketone (100 μM; Molecular Probes Inc.). Recombinant ICE was produced following standard procedures and allowed to partially (5%) autoactivate by incubation at 20° C for 2 hours, followed by storage on ice for a maximum of four hours. Two different

consequences of PT were assessed: a) mitochondrial large amplitude swelling, and b) collapse of the Delta Tm. For determination of swelling, mitochondria were washed and resuspended in CFS buffer supplemented with 2 mM ATP at a concentration of 100 μg mitochondrial protein/10 μl buffer, followed by addition of 90 μM CFS containing 2 mM ATP and recording of adsorption at 540 nm in a spectrophotometer (DU 7400; Beckman Instruments., Carlsbad, CA). After stabilization of the adsorption during a minimum interval of 30 s, the indicated substance was added in a volume of 5 μl. The loss of absorption induced by 5 mM atrectyloside within five minutes was considered 100% of the large amplitude swelling. The Delta Tm was measured using DiOC6 (Levitz et. al., *New England J. Med.*, 338/15, 1074, 1998) (40 nM, 15 minutes at 37° C; Molecular Probes Inc.), after having added the indicated PT inducer (30 minutes, room temperature). Mitochondria were analyzed in an Elite cytofluorometer (Coulter Corp.). All Delta Tm determinations were performed at least three times in each experiment.

EXAMPLE XI

Cell-free System of Apoptosis

Nuclei from cells were purified on a sucrose gradient, washed two times (1,000 g, 10 min, 4° C), and resuspended in CFS buffer. In standard conditions, nuclei (10^3 nuclei/μl) were cultured in the presence of mitochondrial preparations for 90 min at 37° C. Nuclei were stained with propidium iodide (10 g/ml; Sigma Chemical Co.) and the lipophilic dye 5-methyl-bodipy-3-dodecanoic acid (100 nM; Molecular Probes Inc.), followed by cytofluorometric analysis in an analyzer (EPICS Profile II Analyzer; Coulter Corp.). Only membrane surrounded (5-methyl-bodipy-3-dodecanoic acid-labeled) particles were gated. A good correlation between the frequency of nuclei exhibiting chromatin condensation with 4-6-diamidino- 2-phenylindole dihydrochloride (10 μM; Molecular Probes) and hypoploidy with PI has to be obtained. DNA fragmentation was determined by horizontal agarose gel electrophoresis and ethidium bromide staining.

EXAMPLE XII

Western Blot Analysis

Apoptosis inducing factor-mediated cleavage of nuclear substrates was determined by the comparative analysis of SDS-PAGE of HeLa nuclei (5 x 10^6/lane) cultured in the presence or absence of supernatant from Atr-treated mitochondria (10 μg protein/ml, 90 min, 37° C) in the presence or absence of the protease inhibitor Z-VAD.fmk. Western blots of these nuclei were tested for degradation of poly (ADP-ribose) polymerase (PARP) using a monoclonal antibody (C2-10, Guy Poirier, Montreal University, Canada). Cleavage of CPP32 in cells (8 x 10^5 cells/lane) or *in vitro* (10 ng recombinant CPP32+ 10 μg protein of mitochondrial supernatant in 50 μl CFS buffer ± 100 μM Z-VAD.fmk, 15 min at 37° C) was determined by using a polyclonal rabbit antiserum recognizing both CPP32 and the p17 fragment of proteolytically activated CPP32 (Martin et. al., *Cell*, 82:349-352, 1995). Enzymatic activation of CPP32 (100 ng CPP32 + variable amounts of mitochondrial supernatant in 100 μl CFS buffer + 100 μM Z-VAD.fmk, 15 min at 37° C) was detected by adding 1 μM Ac-DEVD-amino-4-methylcoumarin (30 min, 37° C), as described above.

EXAMPLE XIII

Direct Modulation of Immune Cells and Cell Surface Markers by PR-I

The effects of the HIV protease inhibitor on T-cell activation *in vitro* was studied using PBLs from both normal donors (n=16) and HIV-1 infected individuals (n=15). When PBL-cultures were treated for 72 hours with Ritonavir at concentrations similar to those obtained *in vivo* in patients undergoing HIV treatment, an increase in CD25, and CD28 surface molecule expression and a decrease in cellular CD95-ligand (Fas-ligand) was observed on T-cells in both groups, as determined by flow cytometry. Expression of CD95 (Fas) remained unchanged, whereas the number of HLA-DR expressing cells decreased from

about 25% in untreated cell cultures to less than 13% in cell cultures treated with 20 nM PR-I. These dose-dependent effects were more pronounced in the CD4+T-cell subpopulation and differed slightly under various pre-activation conditions (*Blood*, 90/10, Suppl. 1, 2568, 1997).

Further, it has been demonstrated that the expression of CD95 (Fas-receptor) increased on both CD4+ and CD8+ T-cells derived from HIV-1 infected individuals (Sloand et al., *Blood*, 89:1357-1363, 1997), and that preferentially CD4+ cells express active forms of Interleukin-1beta converting enzyme (ICE, also Caspase 1) (Sloand et al., *J. Clin. Invest.*, 101/1, 1-7, 1998). Since these molecules are known to play an important role in mediating T-cell apoptosis (Alderson et al., *J. Exp. Med.*, 181:71-77, 1995; Brunner et al., *Nature*, 373: 441-444, 1995; Dhein et al., *Nature*, 373: 438-441, 1995; Ju et al., *Nature*, 373: 444-448, 1995; Alderson et al, *J. Exp. Med.*, 178: 2231-2235, 1993), we also examined the effect of Ritonavir on CD95 and ICE expression. FACS-analysis of CD4+ T-cells, co-stained intracellularly for ICE protein, revealed significantly lower levels of ICE in the treated cell subsets, independently from their origin (i.e. in both normal donor and HIV infected individual derived cells). These results were confirmed by cell surface staining of phosphatidyl serine on viable cells with Annexin V, that directly correlates with the activation of ICE proteases, therefore representing an early marker for apoptosis.

Interestingly, expression of CD95 (Fas-R) was not directly altered by PR-I treatment *in vitro*. However, the susceptibility of PBLs to CD95 mediated (induced by agonistic mAb CH11, (Yonehara et al., *J. Exp. Med.* 169: 1747-1756, 1989; Trauth et al., *Science*, 245: 301-305, 1989)) apoptosis was markedly decreased as determined by apoptosis and viability assays (TdT, WST-1, PI-staining) (Figures 1-3 and 10a-10d). When PBLs derived from 15 patients with HIV-disease were compared by the same criteria prior to and two weeks after being treated with PR-I, significant decreases in ICE expression, Annexin V

binding, and susceptibility to spontaneous and induced apoptosis were observed.

Similar to the *in vitro* observations, the pattern of cell activation marker expression changed in addition to the increase in total CD4-counts. It is important to point out that these effects were observed at nontoxic (for primary CD4+ T-cells) concentrations of PI. Ritonavir had growth promoting effect on T-cells in short-term (five days) culture (in RPMI 1040, supplemented with 10% heat inactivated FCS, L-glutamine, IL-2) at concentrations of 1.0 nM to 10.0 uM.

These pro-cell-survival effects were even more pronounced when "stress factors" known to be involved in HIV pathogenesis such as TNF-alpha, IFN-alpha, IFN-gamma (Smith et al., *Cell*, 76: 959-962, 1994; Meyaard et al., *Science*, 257: 217-219, 1992; Groux et al., *J. Exp. Med.*, 175: 331-340 1992), or ligands to CD95 (Debatin et al., *Blood*, 83:3101-3103, 1994; Bohler et al, *Blood*, 90:886-887, 1997) were introduced into human PBMC cell cultures (Figure 1). Cells that were exposed to IFN-alpha (50 ng/ml), IFN-gamma (50 ng/ml), TNF-alpha (100 ng/ml), or to the combination of these cytokines were significantly more susceptible to CD95 mediated apoptosis signals (as introduced by the CD95 agonistic mAb CH11, 0.25 μg/ml, 48 hours) than cells that were exposed to the same stimuli but were treated simultaneously with HIV-PR-I Ritonavir. We observed similar effects using other HIV-PR-I in addition to Ritonavir (ICE Inhibitor, Saquinavir, Nelfinavir and Indinavir) that are currently applied in the clinical praxis (Figures 2 and 3). (Inverse correlation of the depicted viability data to the levels of cell apoptosis was confirmed by utilizing the Apoptosis ELISA, Boehringer Mannheim.)

EXAMPLE XIV

Pretease/Proteasome Inhibition Increases the Number of Immune Precursor Cells

Colony forming capacity (CFC) of human bone marrow (BM) derived MNC increased when HIV-PR-I was added to standard methylcellulose culture

assays (Figure 4). As a positive control, caspase-1 (ICE) inhibitors (Patel et al., *FASEB J.*, 10:587-597,1996), known to enhance CFC in culture, were included and compared to the effects of PR-I. While ICE inhibitors had effects at concentrations higher than 100 μM, a significant increase in CFU was obtained with PR-I at concentrations of 10 nM. This data supports the pro-cell-survival effects of HIV-PR-I and its potential use to enhance immune responses.

To see whether these effects would translate into an *in vivo* system, we treated mice with PR-I for three weeks and assessed changes in peripheral white blood cell counts (WBC), as preliminary data had indicated that the effects of PR-I on immune cells were similar in rodents (Figures 5, 7 and 9). As an additional "stress factor", mice were treated with cytoxan (cyclophosphamide) i.p. for one week and the treatment effects were evaluated weekly. Data depicted in the left panel of Figure 5 represent the WBCs of five mice from the treatment group receiving a dose that is equivalent to that used therapeutically in humans, the right graph shows, for comparison, placebo treated mice.

These data clearly provide evidence that HIV protease inhibitors are potent immune modulators, which can change the susceptibility of immune cells to apoptosis as part of their therapeutic benefit. The mode of action is independent of HIV infection, since similar effects were seen in HIV free experimental systems.

EXAMPLE XV

Immunization procedure

BALB/c mice, 6-8 weeks old, were housed in sterilized microisolator cages and maintained on sterile food and water. Mice were grouped at five mice per treatment regimen. Peripheral blood cell counts, serum IgG and weights were determined and reco rded as base line. To assess dose-dependent adjuvant effects of proteasome inhibitors, groups were treated in the initial experiments (based on our preliminary data) with A) 200mg/kg, B) 20mg/kg, C) 2mg/kg proteasome

inhibitor MG123 (Calbiochem), and in a parallel experiment with Ritonavir (Abbott), and D) a placebo (PBS), daily i.p. On day three under treatment, all mice were similarly immunized with an antigen preparation (using purified fully glycosylated HIV-1-bal gp140, 10 μg gp140/mouse) in incomplete Freunds Adjuvant by s.c. injections. One additional positive control group of mice were immunized using complete Freunds Adjuvant to compare these controls to groups A-D. Proteasome inhibitor treatment was discontinued on day five after immunization (Based on our preliminary data, it appears to be most important to change the "set point" of the immune system at the time of immunization.). Subsequent series of experiments determined the optimal duration of treatments that result in strongest immune responses. Additional experiments determined the effect of proteasome modulation on boosting injections. In such experiments, mice were boosted with antigen in incomplete Freunds Adjuvant (IFA) on days 15 and 30 (Figures 6a-6d).

EXAMPLE XVI

Characterization of humoral responses

Blood was collect from the tail veins of vaccinated mice 14, 28, 42, 56, 70, 84, 98, and 112 days after primary immunization. The level of Env-specific IgG was measured in sera separated from these blood samples by ELISA using purified fully glycosylated HIV-1-bal gp140 as described (Abacioglu et. al., *AIDS Research & Human Retroviruses* ,10:371-81, 1994; Moore et. al., *Journal of Virology*, 68:6836-47, 1994). In each ELISA Env-specific mAbs were used as a positive control (Abacioglu et. al., *AIDS Research & Human Retroviruses*,10:371-81, 1994; Moore et. al., *Journal of Virology*, 68:6836-47, 1994; Moore et. al., *AIDS*, 4:297-305, 1990). In addition, these sera were used to follow the development of HIV-1 neutralizing antibodies by the quantitative, linear HIV-1 infectivity assay as described (Nara et. al., *Journal of Virology*, 64:3779-91, 1990; Layne et. al., *Virology*, 189:695-714, 1992; Layne et. al.,

Virology, 65:3293-3300, 1991; Wu et. al., *Journal of Virology*, 69:6054-6062, 1995), incorporating HIV-1_{Bal} (Gurgo et. al., *Virology*, 164:531-6, 1998) to measure homologous neutralization and HIV-1_{IIIB}, HIV-1_{RF} as well as primary HIV-1 isolates (from the AIDS Repository, NIAID) to measure clade-specific and cross-clade neutralization (Nara et. al., *Journal of Virology*, 64:3779-91, 1990; Layne et. al., *Virology*, 189:695-714, 1992; Layne et. al., *Virology*, 65:3293-3300, 1991; Wu et. al., *Journal of Virology*, 69:6054-6062, 1995).

EXAMPLE XVII

Characterization of CD4+ T cell responses

To measure T cell proliferation, each MNC preparation was stimulated with purified fully glycosylated HIV-1_{Bal} gp140 (Intracel Inc.) and proliferation was quantitated by ^{3}H-TdR incorporation. Each proliferative assay included a mitogen (Con A) control, an ovalbumin control, and a dose-response curve to Env (0.01-10 μg/ml). Supernatants were collected 72 hours after antigenic stimulation and chemokines MIP-1α and MIP-1β, and cytokines IL-4, IL-5, IL-6, IL-10, IL-12 and IFN-γ were measured by ELISA using commercially available reagents. Env-specific T-cells were further characterized by chemokine- and cytokine-specific ELISPOT assays for MIP-1α, MIP-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IFN-γ production after enrichment of CD4+ T cells using commercially available monoclonal antibodies (R&D Systems and Pharmingen).

In addition, MNCs are cultured in CM only, or CM containing fully glycosylated HIV-1_{Bal} gp140 at 0.1-10 mg/ml. The culture plates were incubated for 24 hours at 37° C in 5% CO_2. After stimulation, CD4+ and CD8+ T-cells were isolated by flow cytometry and no fewer than $5x10^5$ of the purified cells were placed directly into Trizol^R reagent and cDNA was synthesized; the resultant samples were then used in quantitative-competitive (QC) PCR reactions to evaluate the relative levels of chemokine and cytokine cDNA sequences. MIP-1α, MIP-1β, RANTES, TNFα, IL-2, IL-4, IL-5, IL-6, and IFN-γ sequence

specific primers are described. Each QC PCR reaction was conducted in parallel with the same reactions containing of control plasmid DNA at a range of defined concentrations, which encode truncated MIP-1α, MIP-1β and RANTES cDNA sequences and served as competitive sequences (R&D Systems and National Biosciences Inc). The PCR fragments were separated by agarose gel electrophoresis, strained with ethidium bromide and scanned using a BioRad UV densitometer. The results were expressed as arbitrary mRNA units.

EXAMPLE XVIII

Characterization of CD8+ T cells

To characterize HIV-specific chemokine-secreting CD8+ T-cell responses, a further group of mice were immunized as above. On days 7, 14, 28, 42, 56, and 70 after immunization, groups of five mice were sacrificed and MNCs from the spleens were prepared. The MNCs were divided into total, CD4+ T cell-depleted and CD8+ T cell-depleted cells, and were cultured in (i) medium only, (ii) medium containing PHA, (iii) medium containing BC-*lacZ* fibroblasts expressing β galactosidase (Aggarwal et. al., *Journal of Experimental Medicine*;172:1083-90, 1990) or (iv) medium containing BC-*env* fibroblasts expressing gp160 (Abimiku et. al., *AIDS Research & Human Retroviruses*, 11:383-93, 1995). The stimulator cells were titrated in the range of 1000 to 500000 cells per well. The cells then were incubated for 72 hours at 37°C in 5% CO_2 and supernatants were collected every 24 hours and stored at -80°C. These supernatants were used to quantitate the levels of MIP-1α, MIP-1β, TNF-α, IL-2, IL-4, IL-5, IL-6 and IFN-γ by ELISA (see above). In addition, after stimulation CD8+ T cells were isolated from total MNC cultures by flow cytometry to obtain a minimum of $5x10^5$ cells, which were placed directly into Trizol^R reagent and cDNA was synthesized; QC PCR reactions was used to evaluate the relative levels of chemokine and cytokine cDNA as described.

EXAMPLE XIX

Class I restricted CTL responses

After five days of such stimulation, HIV-specific CTL activity in spleen cells was also measured. CTL activity was measured using targets ^{51}Cr-labeled (i) BC-*lacZ* fibroblast or (ii) BC-*env* fibroblast (Chada et. al., *Journal of Virology*, 67:3409-17, 1993; Fujihashi et. al., *Journal of Immunology*, 145:2010, 1990). When killing was observed, the effector cells were phenotyped using standard methods applied by our group previously (Aggarwal et. al., *Journal of Experimental Medicine*;172:1083-90, 1990). For quantitative comparisons, CTL responses were expressed in lytic units/10^6 cells. To evaluate if the immune enhancing effects of proteasome modulation could be utilized for therapeutic vaccines in HIV disease, an HIV transgenic mouse model was used (Dickie et. al., *Virology*, 185:109-19,1991). These animals show a number of phenotypic changes and develop functional defects that are similar to pathophysiological changes that are commonly observed in HIV disease. These changes include chronically and significantly elevated levels of proinflammatory cytokines, as well as increased levels of apoptosis in a variety of tissues. Under these conditions, modulation of immune responses differed from that of a normal organism. The treatments and immunizations were performed, and immune response assessment was conducted as described above, and results were evaluated under similar criteria as described for Balb/C mice.

EXAMPLE XX

Immune Enhancing Effects of HIV-PR-I *in vivo* Utilizing Established Rodent Models

Immunizing mice with antigen and assaying for PR-I mediated differences in specific humoral immune responses demonstrated the immune enhancing effects of HIV-PR-I *in vivo* (Figures 6a-6d). Further, this was accomplished by assessing the effect of HIV-PR-I on HIV gene-product-induced cytokine

disturbances and pathophysiological changes that are displayed in HIV transgenic (gag-pol-deleted) rats.

An established immunization protocol in mice was used to assess whether HIV PR-I enhances the immune responses in an HIV-free system. Immune enhancing effects of "additives" like IL-12, or GM-CSF, co-injected with antigens, are well known (Fauci et al., *Science*, 262: 1011-1018, 1993). Similar effects may be mediated by HIV-PR-I. Since our preliminary data indicated that murine cells are sensitive to the modulation by PR-I that we have primarily observed in human cells, we used a murine *in vivo* model to see whether parallel administration of Ritonavir with an antigen resulted in a more effective immunization, as determined by specific humoral immune responses. For immunizations, 6-8 weeks old female BALB/c mice were grouped at 5 mice per treatment regimen. Peripheral blood cell counts, serum IgG and weights were determined and recorded as "base line". To assess dose-dependent effects, groups were treated with A) 200 mg/kg, B) 20 mg/kg (human therapeutic equivalent), C) 2 mg/kg Ritonavir and D) a placebo (PBS), twice a day via oral application (gastro lavage). On day 3 under treatment, all mice were similarly immunized with an antigen preparation (5 μg ovalbumin/mouse) in incomplete Freunds Adjuvant by s.c. injections, and on days 13 and 23 mice were boosted with antigen in incomplete Freunds Adjuvant. One additional positive control group of mice were immunized using complete Freunds Adjuvant to compare these controls to groups A-D. Nine days after each antigen application, eye-bleed derived serum were obtained from all animals and assayed for specific antibodies to the antigen utilizing ELISA techniques. This approach allowed us to get measurable antibody levels in the control (placebo) group and possibly statistically significant and dose dependent increases in the treatment groups A-C, when compared to C. All samples from individual mice were determined in duplicate determinations, and statistical significance analysis for the differences between groups was applied.

Further studies were conducted to determine optimal dosing and specific immunization conditions for different HIV derived antigens that under current condition are poorly antigenic. Subsequent experiments defined the levels of cellular immune response utilizing standard CTL assays. Determination of viral suppressive factors (VSF) that are known to affect HIV replication were also conducted. These *in vivo* results provided valuable information on the specific cellular elements affected by HIV-PR-I and may lead to the identification of new alternative drugs having similar immune enhancing effects. In the event new candidates should be identified by *in vitro* studies, the animal model described above would then serve to evaluate their efficacy as immune modulators. We determined if such immune modulation can be specifically applied to augment candidate vaccine testing in primates and/or humans.

VERTEBRATE ANIMALS.

Mice were maintained in the IHV Animal Facility. The animals were housed, cared for, and used strictly in accordance with the NIH Guide for the *Care and Use of Laboratory Animals* (NIH Publication No. 85-23. 1985). This institution has an Animal Welfare Assurance on file with the NIH Office for Protection from Research Risks (OPRR), Assurance Number A3200-01. The Animal Facility provided accredited veterinary support and resources for the murine studies. This included expertise for the care and handling of the animals, standard operating procedures for the inoculation of animals and for the collection of specimens, including tissue samples. In addition, the IHV animal facility is equipped with surgical rooms, laboratory equipment and biological safety cabinets, thereby allowing both Biosafety Level 2 and 3 on-site manipulation of animals.

Primary veterinary care was provided by a full-time, specially trained, laboratory animal veterinarian, who was responsible for the care and manipulation of all study animals affiliated with this project. The laboratory animal veterinarian

met with the investigators to assist in the design and review of all animal protocols, schedule and supervise the execution of study protocols, and reviewed all study records for protocol completion.

To ensure a comfortable and constant environment, the temperature in the animal rooms was maintained at 23-25° C and humidity is maintained at 50-20%. The temperature and humidity levels were monitored continuously via internal sensors tied to a central computer monitoring system. The system was also monitored on a 24-hour basis at a console in the office of the Facility Manager. Audible and Visual alarms alerted the Manager if the temperature or humidity drifts outside the desired parameters. To maintain clean air in the animal rooms, negative air pressure was maintained in these rooms. Air pressure gradients were continuously maintained with airlocks and positive air pressure flow to the hallways. The air handling system has independent capability control units designed to achieve Biosafety Level 2 and 3 and to maintain the airflow throughout the animal facility. The rate of non-recirculated air exchange in each animal module was 15-20 times per hour. To mimic natural day and night cycles, lighting was controlled automatically to provide twelve hours each of darkness and light. The light cycle period was adjustable if needed.

Procedures for minimizing stress and discomfort included anesthesia (metaphane) during immunizations and any other invasive procedure. No restraining devices were used throughout the proposed study.

Carbon-dioxide inhalation was used as a means for euthanasia in accordance with institutional guidelines.

EXAMPLE XXI

Immune Response to HIV gp140 Antigen *in vivo* is Increased by Adjuvant Effects of Proteasome Inhibitor Treatment

To see whether these immune modulating effects of proteasome inhibitors would translate into an *in vivo* system, we first assessed different drug

concentrations and performed toxicity testing over a time period of three weeks. In confirmation of data reported by others (Andre et. al., *PNAS*, 95:13120, 1998; Harding et. al., *J. Immunol.*, 155, 1767, 1995; Di Cunto et. al., *Science*, 280, 1069, 1998; Conner et. al., *J. Pharmacol. Exp. Ther.*, 282, 1615, 1997; Marienfelder et. al., *Eur. J. Immunol.*, 27, 1601, 1997), we found that proteasome inhibitors had similar effects on cytokines in rodents, as compared to human cells (*see* Example XX). These data provided the basis for subsequent immunization experiments that were performed to assess whether the changed immune milieu would result in a different immune response to HIV antigen. Systemic administration of Ritonavir as an immune adjuvant (Figure 6a) and local co-injection of antigen and Ritonavir as an adjuvant (Figure 6b) resulted in specific humoral (antibody) immune responses induced by immunizations. It can be seen from these results that an increased amount of neutralizing antibodies were detected in the serum of mice (group of 5) that received co-treatment with the PR-I (Ritonavir). As depicted in Figures 6c and 6d, mice that received proteasome inhibitor treatments during immunization with recombinant HIV gp140 developed significantly higher titer of specific and neutralizing antibodies than the control groups as measured by ELISA, 30 days after a single injection of Ag in IFA.

The experiment illustrated in Figure 6d represents data from pooled serum of five mice per treatment group. Results have been reproduced twice in independent experiments, in one of which ovalbumin was used as antigen.

Similar to *in vitro* effects of proteasome modulation, enhancement of specific Ab titers was dependent on the dose used for treatments, but, most interestingly, also on the duration of treatment. It appeared that the "set-point" of an immune system at the time of immunization affects markedly the subsequent immune response. Thus, it was necessary to further characterize the immune enhancing properties of proteasome inhibitors and to better determine the conditions necessary to apply this strategy in immunization protocols.

EXAMPLE XXII

Effects of HIV-PR-I in Primary Human and Rodent Cell Culture Systems

Flow cytometry in conjunction with surface marker staining showed the changes in phenotype and cell activation of the different cell subsets in response to HIV-PR-I treatment of primary human and rodent cell cultures. Specifically, HIV-free primary cell culture systems were utilized to obtain information. PBMCs were exposed in culture to different HIV-PR-I so as to determine dose-dependent effects on cellular activation as measured by cell surface marker multicolor FACS analysis. Additionally, we measured changes in the resistance of HIV-PR-I treated human and rodent cell cultures to "stress factors" that are typically associated with HIV infection utilizing metabolic assays, cell death and apoptosis assays (Figures 2, 3, 5, and 7).

Further, HIV-PR-I may enhance the production of cytokines or regulate the cellular susceptibility for cytokine signals that augment immunizations, such as IL-2, IL-12, or GM-CSF. It is possible that immune suppressive cytokines (such as IFN-alpha, TNF-alpha and the like) or their effects (known to be involved in HIV pathogenesis) are blocked by PR-I. In that context, some of our *in vitro* data indicated pro-hematopoietic effects that could be due to regulation of cytokine production by PR-I (Figures 4, 16a-16b, 17, 19b and 29).

Additionally, we assessed cellular markers including: a) CD3, CD4, and CD8 (for identification of T-cell subsets); b) CD14 and CD86 (for identification of monocytes); and c) CD16 and CD56 (for identification of NK cells). Markers of activation that were assessed in cell subsets (when appropriate) include: CD11a, CD25, CD26, CD28, CD38, CD45RO, CD45RA, CD62L, CD69, CD95, Fas-ligand, CDw119, IFN-receptors, TNF-alpha receptors, and HLA-DR. Cell culture supernatants were screened for cytokine profiles, first for known immune augmenting cytokines such as IL-2, IL-12, GM-CSF, and for known immune suppressing cytokines such as IFN-alpha, TNF-alpha, Fas-ligand (Katsikis et al.,

J. Exp. Med., 186/8, 1365-1372, 1997), using ELISA technique. Since some chemokines were identified as viral suppressive factors in HIV infection and may therefore play a role in AIDS pathogenesis, commercially available chemokine assays were included in the evaluation of factors that are affected by PR-I treatment. To confirm or reevaluate these data, differential mRNA analysis on cells derived from differently treated cultures were performed.

For the rational utilization of the immune enhancing phenomenon displayed by HIV-PR-I in vaccination protocols, a detailed characterization of the drug's impact on immune cells was conducted. Based on our preliminary data, HIV-PR-I inhibited apoptosis. The regulation of immune cell apoptosis as a concept for a therapy that augments vaccination appeared reasonable since HIV infected cells escape apoptosis mediated immune surveillance; significantly increased levels of apoptosis in lymphoid organs and T-cells correlate with HIV disease progression; and highly-HIV-antigen-specific T-cells are most susceptible to apoptosis and get depleted prematurely.

Since our preliminary data suggested that immune augmenting effects of PR-I are more significant under the influence of factors that were suggested to be involved in the HIV associated immune suppression, we tested if *in vitro* induction of such immune suppressive cytokines (as described above) was blocked by the drugs. In different experimental settings, we investigated whether the effects of IFN-alpha, IFN-gamma, and TNF-alpha on the induction of apoptosis, were altered by PR-I. Cell apoptosis and viability were determined using TdT (terminal transferase) assays, combined with PI (propidium iodide) measurements. Metabolic rates were measured in automated WST-1 (similar to MTT) assays using microtiter plates and photometric assessment.

After identifying the best ways to detect PR-I treatment-introduced changes in cells with the highest accuracy, it was necessary to select a cell line model to circumvent difficult factors of variation associated with primary cells. Working

with clonal populations allowed cell cycle and signal transduction pathway studies. For the identification of a suitable cell line we focused on fast and easy to perform tests that allow a high screening throughput, such as FACS based Propidium Iodide/Annexin V-, ICE-, and CPP32-measurements of differently treated cells. All of these assays are commercially available and were successfully used in our laboratory (Sloand et al., *Blood*, 89:1357-1363, 1997; Sloand et al., *J. Clin. Invest.*, 101/1, 1-7, 1997) and are therefore not described herein. Cell lines that were evaluated include: PM1, Jurkat, MOLT3, SupT1, HUT78 (for lymphocytoid cells), U937, THP1 (for monocytoid cells), KG1, KG1a, and K562 (for hematopoietic precursor cells). As soon as suitable cell lines were identified, our studies shifted toward the characterization of cellular pathways that are altered by PR-Is. The activation marker data obtained in previous experiments provided important hints regarding where to look (what cellular pathways) to identify the specific mechanism(s) responsible for the observed modulations. It was also determined: a) if controlled apoptosis inhibition can restore or preserve immune cells and their functions, and b) at what stage of the apoptotic processes the interference should take place.

EXAMPLE XXIII

Further Assessment of Cellular Pathways and Identification of Elements That Are Modulated by Protease Inhibitor Treatment

After identifying a suitable cell culture model, the different pathways that could be involved in immune augmentation by PR-I were studied in detail. Our first focus was the assessment of changes in apoptosis pathways. It is currently assumed that the apoptotic process can be divided into at least three functionally distinct phases. During the heterogeneous initiation phase, cells receive the death-inducing stimulus via certain receptors such as the TNF receptor or Fas/APO-1/CD95, shortage of obligatory growth factors, oxygen or metabolic supply, or subnecrotic physical and chemical damage. The biochemical events

participating in the initiation phase constitute "private" pathways in the sense that they depend on the lethal stimulus. It is only during the subsequent phases that these initiating events are translated into a regular common pattern of metabolic reactions. The common pathway can be subdivided into an initial effector phase, during which the "central executioner of apoptosis" is still subject to regulatory mechanisms, and a later degradation phase, beyond the "point of no return", during which catabolic enzymes become activated in an irreversible fashion. During the degradation phase the morphology and characteristic biochemistry of apoptosis (e.g., step-wise DNA fragmentation, and specific proteolysis of cytoplasmic and nuclear substrates) become manifest (Wertz et. al., TIBS, 21:359-364, 1996; McConcey et. al., *Trends Cell Biol.*, 4:370-375, 1994; Martin et. al., *Cell*, 82:349-352, 1995; Martins et. al., *Trends Cell Biol.*, 7:111-114, 1997).

Two nonexclusive mechanisms have been proposed to intervene as central executioners of the apoptotic effector phase. On one hand, it appears that apoptosis is associated with the critical activation of a family of specific proteases that include interleukin-1 converting enzyme (ICE1/caspase 1), CPP32 (Yama/Apopain/caspase 3), and other proteases homologous to the Caenorhabditis elegans protein Ced-3. On the other hand, the disruption of the mitochondrial inner transmembrane potential (delta Tm) marks a point of no return for the apoptotic cascade. Mitochondria that undergo permeability transition (PT) or products derived from these organelles induce chromatin condensation and DNA fragmentation in cell-free systems of apoptosis (Susin et. al., *J. Exp. Med.*, 186/1, 25-37, 1997). Our focus was to determine which of the apoptosis pathways is altered by HIV PR-I. To achieve this, cell metabolic labeling, and immune precipitation assays, as well as kinase assays were performed.

The detailed assessment and characterization of the initially herein described phenomenon provided important information in the development of new means to target immune modulations that may increase the effectiveness of

vaccinations. The data served to evaluate the conscious utilization of HIV-PR-I for immune augmentation. This strategy was re-tested in animal models and if successful applied to humans as the drugs are already approved. The project also provided key information for subsequent, highly specific clinical research, where the proposed combination of antiviral with apoptosis-preventing treatment for immune restoration in HIV-disease needs to be evaluated (Figure 8). Improved immune responses facilitated more successful immunization regimens for a variety of pathogens in immune compromised individuals.

EXAMPLE XXIV

HIV-1 Protease Inhibitor Modulates Activation of Peripheral Blood CD4+ T-Cells and Decreases Their Susceptibility to Apoptosis *In Vitro* and *In Vivo*

Ritonavir and cell activation

To assess the effects of Ritonavir on cell activation we first determined the toxicity of the drug in HIV-free cell cultures. It has been reported that Ritonavir is not toxic at concentrations up to 20 μmol/l in cell cultures and that HIV inhibition is significant (IC50) at concentrations as low as 1 nmol/l (Norvir, Ritonavir. Product monograph. Abbott Laboratories, North Chicago, Ill. Package Insert, 1997). The concentration required to reduce HIV-protease activity (Ki) by 50% is lower than the IC50 (as low as 0.1 nmol/l) (Flexner C: HIV-protease inhibitors, *New England J. Med.*, 338:1281, 1998; Kempf et. al., *PNAS*, 92:2484, 1995). Our data obtained with PBMC long-term cultures over 15 days indicated marked inhibition of cell growth and viability at concentrations higher than 50 nmol/l as determined in WST-1 assays (Figure 7). Increased cell numbers, as compared to untreated controls, were measured in cultures treated with lower concentrations of , detectable at concentrations as low as 0.5 nmol/l. These results were confirmed by cell counts using Trypan blue exclusion. By comparison, plasma levels that were determined in clinical studies ranged from 3 μmol/l to 15

μmol/l. It was also estimated that only about 1% of the drug is bio-available, mainly due to drug / plasma protein interactions (Barry et. al., *Clin. Pharmacokinet.*, 32:194, 1997). Tissue levels effective in lymphoid organs, for example, have not been specifically measured, but could be at a range where we detected increased survival of cells in our *in vitro* systems.

Ritonavir and apoptosis

Since enhanced immune cell apoptosis is thought to be a major factor in AIDS pathogenesis, we investigated whether Ritonavir would protect activated uninfected T-cells from dying. Factors that have been shown to be involved in mediating apoptosis and that were found to be elevated in HIV disease include IFN-alpha, IFN-gamma, TNF, and CD95 (Fas, Apo1)-ligand (Flexner C: HIV-protease inhibitors. *New England J. Med.*, 338:1281, 1998). We exposed donor-derived, preactivated PBMC to IFN-alpha, IFN-gamma, and TNF at concentrations that we previously found increased death receptor expression in culture without exerting toxicity (Maciejewski et. al., *J. Immunol.*, 153:4303, 1994; Zella et. al., *Blood*, 91:4444, 1998). Ritonavir (10 nmol/l) was supplied to the cultures. After 3 days, CD95 agonistic mAbs were added, and 16 hours later, cell viability was measured to estimate the amount of CD95 related apoptosis. Marked differences were evident between cultures treated for three days with Ritnoavir and cytokines, and cultures treated with cytokines alone (Figure 1). Effects of cell death induction as well as inhibition by the HIV-PR-I were dose-dependent, as illustrated by a cross-titration with the CD95 agonistic mAb CH11 (Figure 9). These experiments provide evidence that Ritonavir exerts protective effects against cell death-mediating "stress factors" that are commonly associated with HIV-disease.

EXAMPLE XXV

Reduced Apoptosis Correlates with Decreases in Caspase-1 Expression and Caspase-3 Activity in normal cells

In previously reported experiments we have used cell-surface-AnnexinV-staining as a sensitive marker to identify cells that are in the process of apoptosis (Sloand et. al., *J. Clin. Invest.*, 101:195, 1998)(See Example V). In similar experimental settings, we have measured intracellular expression levels of the cysteine protease Caspase-1 (Casp1, or IL-1-beta-converting enzyme, ICE) and determined the utility of these flow cytometry based assays for cell activation assessment in HIV-patient-derived PBMC fractions. In confirmation of results reported by other laboratories (Debatin et. al., *Blood*, 83:3101, 1994; Katsikis et. al., *J. Exp. Med.*, 181:2029, 1995), we determined that increased detection of both Casp1 and AnnexinV correlated with abnormally high levels of activation induced apoptosis in these specimens (Sloand et. al., *Blood*, 89:1357, 1997; Sloand et. al., *J. Clin. Invest.*, 101:195, 1998). We were interested in whether the *in vitro* effects of Ritonavir would relate to changes detectable with the Casp1 and AnnexinV assays. When PBMC cultures were activated and treated for three days under the standardized conditions as described above, flow cytometric analyses revealed that PR-I treated cells expressed less Casp1 (Figure 10 panel A) and showed lower levels of AnnexinV stain (Figure 10 panel B) than PBMC subsets in control cultures. These changes were detectable at Ritonavir concentrations ranging from 0.5 nM to 50 nM, supporting our data obtained in the cell viability assays. Since cell viability assessment may also reflect growth inhibitory effects, we further confirmed that decreases in cell number correlated with increases in apoptosis rates in respective cultures, by utilizing a specific TdT (terminal deoxynucleotidyl transferase) and TUNEL assays (Figure 3).

One of the main effector of caspases in several apoptosis pathways is Caspase-3. Since CD95 triggering leads to an increase in Caspase-3 activity, we investigated the effect of Ritonavir treatment on this enzyme's activity in PBMC cultures, utilizing a spectro-fluorometric assay. Significantly less Caspase-3 activity was detected in cytoplasmic extracts of activated PBMC cultures that were treated with Ritonavir and exposed to anti-CD95 (CH11) mAb (under the standard conditions described above) when compared to untreated control cells (Figure 11a). Higher Caspase-3 activity in cultures that were not treated with Ritonavir peaked one hour after CD95 triggering and correlated to lower cell viability detectable after 16 hours. In immortalized (tumor) cells, specifically human promonocytic leukemia (U937) cells, Ritonavir increased caspase-3 activity and death by apoptosis suggesting that Ritonavir has the ability to selectively effect cellular pathways, for example, inducing apoptosis in tumor cells or inhibiting the activation of nuclear factors (Figure 11b and 22).

EXAMPLE XXVI

Ritonavir Decreases CD95 and CD95 Ligand Expression on T-cells

Since both CD95-dependent and independent induction of T-cell apoptosis in AIDS were reported (Ashkenazi et. al., *Science*, 281:1305, 1998), we assessed the effect of Ritonavir on CD95 and CD95-ligand levels *in vitro*, utilizing flow cytometry. After 3 days of treatment of PBMC under the conditions described, a slight decrease in CD95 expression was associated with the application of Ritonavir (data not shown). CD95-ligand expression on T-cells was markedly decreased in cultures treated with 10 nM Ritonavir. These effects were detectable in both the CD4+ and CD8+ T-cell subset, but more pronounced in the CD4+ cell population (Figure 10 panel C).

In addition, we examined the effects of Ritonavir on the CD95 ligand apoptosis mediating system. It can be seen in Figure 10d that CD95-ligand expression on T-cells was markedly decreased in cultures treated with 10 nM

Ritonavir. These effects were detectable in both the CD4+ and CD8+ T-cell subset, but more pronounced in the CD4+ cell population. Ritonavir mediated decreases in Fas-ligand protein-content in lysates of PBMC were measured by Western blot (Figure 10E) and densitometrical quantification of specific protein bands. Figure 10E depicts a Western blot of fas-ligand protein. It can be seen that when Ritonavir decreases proteasome activity, there is a subsequent increase in ubiquitinated proteins (which are normally processed by proteasome complexes) and a decrease in fas-ligand expression.

EXAMPLE XXVII

Release of Inflammatory Cytokines Inhibited by Ritonavir

Clinical evidence suggests that anti-retroviral combination therapy can also lead to the reduction of "harmful" cytokines (Lederman et. al., *J. Infect. Dis.*, 178:70, 1998; Flexner C: HIV-protease inhibitors, *New England J. Med.*, 338:1281, 1998; Autran et. al., *Science*, 277:112, 1997). Since it was postulated that abnormally high levels of TNF as observed in HIV-infected individuals contribute to immune cell dysfunction and loss (Fauci et. al., *Science*, 262:1011, 1993; Maciejewski et. al., *J. Immunol.*, 153:4303, 1994; Oyaizu et. al., *Blood*, 84:2622, 1994), we investigated if Ritonavir affects the production of this cytokine. Supernatants from activated PBMC cultures were collected and TNF was measured by ELISA. Ritonavir treatment inhibited the release of the cytokine in a dose- and time-dependent manner (Figure 12). This inhibition was reversible, as freshly separated PBMC cultures that were treated with Ritonavir for only one day responded three days later to TCR activation (with anti-CD3 mAbs) by producing TNF levels similar to untreated controls (data not shown).

Elutriated monocytes that were incubated with Ritonavir for one hour prior to induction of TNF by LPS produced significantly less of the pro-inflammatory cytokine in culture supernatants as measured by ELISA (Figure 13). When TNF-alpha was induced by IFN-gamma (50 ng/ml) in monocyte cultures using

comparable experimental conditions, Ritonavir also decreased the release of TNF into supernatants, with more than 60% inhibition seen at a concentration of 10 nM (data not shown).

EXAMPLE XXVIII

Reversible, Partial Proteasome Inhibition Results in the Differential Regulation of Cytokines and Chemokines That Regulate Inflammation, Neovascularization and Immune Responses

Whereas complete inhibition of the major cytosolic protease, the proteasome, is deadly to a cell within hours, partial and reversible inhibition has a variety of consequences that are relevant for immune functions. Cytosolic antigen processing and subsequent presentation by MHC-I are functions that classically have been linked to the proteasome pathway (Harding et. al., *J Immunol.*, 155, 1767, 1995). Other proposed functions include cell cycle regulation (Di Cunto et. al., *Science*, 280, 1069, 1998) and regulation of inflammation (Conner et. al., *J. Pharmacol. Exp. Ther.*, 282, 1615, 1997).

In the following examples, evidence is presented, for the first time, to show that partial and reversible proteasome inhibition results in enhanced immune responses *in vivo*. These results support the idea that proteasome inhibitors, rationally applied to vaccination, have immune enhancing properties.

Elutriated monocytes obtained from normal volunteers were treated with the proteasome inhibitor (PI) Ritonavir at a concentration of 10 nmol/l and compared with untreated controls. Cells were harvested and mRNA was extracted for analysis with optimized Rnase protection assay systems (PharMingen) that allow a truly quantitative analysis of different mRNA species. Data in Figures 12 and 14 are depicted as changes in mRNA levels as calculated in comparison to mRNA levels that were obtained from untreated monocyte control cultures. These data confirm PI-mediated changes in cytokine and chemokine levels that were detected by ELISA in culture supernatants. In the analysis of levels of TNF,

supernatants were collected from cultures and TNF protein was quantified by ELISA (Figure 12).

Effects of Ritonavir on IL-8 production

To determine the effects of Ritonavir on IL-8 production, activated, proliferation human vascular endothelial cell (HUVEC) cultures were treated with the PI Ritonavir (30 nanomol/l) and, after two days, supernatants were collected for ELISA analyses of IL-8 production. Comparison with IL-8 levels of untreated cultures indicate a decrease in levels of this cytokine by the PI-treatment (Figure 21). This data supports the discovery that protease inhibitors have anti-inflammatory effects that influence endothelial cell activation and proliferation, thus inhibiting mechanisms that also can lead to tumor neovascularization. This is demonstrated in Figures 15-18.

EXAMPLE XXIX

Proteasome Modulation Improves the Effectiveness of Vaccinations

Differential regulation of cytokines and chemokines that affect immune cell trafficking and immune responses provided one possible explanation for immune adjuvant effects of systemic PI-treatment *in vivo*. We hypothesized that such modulation would affect the "set-point"of an immune response to specific HIV antigens (Figures 6a-6d). We provide here additional data on immunization experiments. Mice (Balb/c, five mice per treatment group) were immunized with 10 μg HIV gp140 alone, in combination with Alum (Pierce), or (300 μg) in single s.c. injections . In a different immunization group, mice were pretreated for five days with Ritonavir systemically by i.p. injections of 300 μg/mouse/day. PI-treatment in this group was continued for additional five days post immunization (Figures 6a-6d). Serum for measurements of Ig levels specific for gp140 was collected three weeks after immunization and antibody titers were determined by ELISA. Systemic application of PI during the time of

immunization and initiation of immune response resulted in a marked increase of the effectiveness of vaccination.

EXAMPLE XXX

Engraftment of human cord blood in SCID/NOD mice

In vivo models of engraftment of human bone marrow cells have been established using irradiated mice carrying the SCID mutation (Uckun et al., *Blood*, 88:1135, 1996; Lapidot et al., *Blood*, 88:2655, 1996). To examine the influence of protease inhibitor treatment on the outcome of human bone marrow transplantation in vivo, we engrafted primary human cord blood derived cells into irradiated severe combined immunodeficient/nonobese diabetic (SCID/NOD)mice and tested the effect on engraftment, proliferation, and dissemination of human cells in these mice. The results show that protease inhibitor treatment effectively improves the outcome of hematopoietic cell transplantation in vivo.

Methods

Mononuclear cells (MNC) were prepared from human cord blood using dextran sedimentation of erythrocytes and density centrifugation as previously described (Iversen et al., *Blood*, 88:2634, 1996). For transplantation we used female SCID/NOD mice aged 4 to 6 weeks that were bred and maintained in a specific pathogen-free environment. On day 1 (immediately before transplantation of cells), all mice were irradiated with 200 cGy to facilitate engraftment of human cells. We then injected 10^7 MNC intravenously on day two. Groups of ten transplanted mice were allocated to receive treatment for 1 week with either saline (placebo), or Ritonavir (30 mg/kg), or recombinant human interferon gamma (5 ug/kg) daily by intra peritoneal injections. To determine engraftment of human cells into SCID/NOD mice the animals were killed with an overdose of pentobarbital (intraperitoneally) and different cellular compartments were analyzed.

A quantitative and qualitative assessment of human cell contents in the recipient mouse bone marrows was performed with Flow cytometry. Phenotyping of engrafted human cells was performed by staining cells collected from the femoral bone marrow of transplanted mice with an anti-CD45 MoAb and sorted with flow cytometry. These human-specific CD45+ cells were then stained with MoAbs (Becton Dickinson) raised against the following antigens and analyzed with flow cytometry: CD34 (hematopoietic progenitors, stem cells) CD38 (activation marker, absence indicates more primitive stem cells), CD33 and CD14 (myeloid markers), CD3 and CD19 (lymphoid markers). Appropriate isotype-matched IgG were used as a negative controls. Analyses were performed on an EPICS-Profile II Flow Cytometer (Coulter Electronics, Hialeah, FL).

To determine function and colony forming capacity of engrafted human progenitor cells, hematopoietic progenitor assays were performed. Femoral bone marrow cells (10^5 cells per ml culture meduim) were grown for 14 days in methylcellulose under conditions that selectively promote colony formation by human, but not by mouse, progenitor cells (Sirard et al., *Blood*, 87:1539, 1996).

Statistics

Values are given as the means and standard error of the mean (SEM). Differences were evaluated with Kruskal-Wallis test including Bonferroni's test when appropriate. Two-tailed tests were used. Statistical significance was assumed for $P < .05$.

Results

Human hematopoietic progenitor cells engraft and proliferate within SCID/NOD mice as assessed in a long-term engraftment assay, six months after transplantation. Phenotyping of bone marrow derived cells indicated that 24 ± 8% of cells were of human origin (i.e. hCD45+) and that some progenitor cells (CD34+) had been maintained in the untreated mice. In transplanted mice that had

been treated with hIFN-gamma, a known suppressor of hematopoietic cell maintenance and function, less than 10% of human cells were retrieved. Long-term engraftment in Ritonavir-treated mice was found to be much higher than in both control groups, as high as 49 ± 14, thus reconstituting a major fraction of the cells in the mouse bone marrow (Figure 24, flow scattergram, representative example of each group: upper left panel - not transplanted, lower left panel - transplanted without treatment, upper right panel - transplanted with Ritonavir treatment, lower right panel - transplanted with IFN-gamma treatment).

The degree of engraftment was tested functionally by plating cells from the bone marrow of transplanted mice in semisolid medium under conditions that selectively support growth of human progenitor cells (Table 1). These data indicated that protease inhibitor treatment immediately after radiation, during the initial week of engraftment resulted in a significant higher number of human cells that had better hematopoietic potential than cells that were transplanted without PI-conditioning. The effects of Ritonavir on irradiated mice are depicted in Figures 19a-b and 20. As a negative control, we used bone marrow cells from untransplanted mice and these cells did not form any colonies.

Table 1

Treatment	Engrafted Animals	Human Cell Engrafted	Human Stem Cells (CD34+)	CFU Capacity *in vitro*
No transplant	0/0	0%	0%	0%
Transplanted + Placebo	6/10	24 ± 8%	4 ± 0.5%	100%
Transplanted + Ritonavir	9/10	49 ± 14%	7 ± 2.3%	168%
Transplanted + hIFN-gamma	5/10	7 ± 4%	0.4 ± 0.3%	23%

EXAMPLE XXXI

Protease inhibitor directly inhibit AIDS related malignancies and other cancer *in vitro* and *in vivo*

Kaposi's Sarcoma (KS) is a highly angiogenic tumor of endothelial cell origin, characterized by spindle shaped cells and infiltrates of immune cells. Inflammatory cytokines, as well as viral gene products such as HIV Tat and HHV8 orf74 contribute to the development of KS lesions associated with HIV-1 infection.

We have previously reported that the HIV protease inhibitor Ritonavir modulates *in vitro* immune cell activation and susceptibility to apoptosis (programmed cell death) in HIV-free culture systems (*Blood*, 90/10, suppl. 1,2568: 1997). Based on our data, we hypothesized that Ritonavir exerts its therapeutic benefits at least in part by direct modulation of apoptosis that is independent from HIV protease inhibition *(Blood,* 94/3, 1021: 1999, and *Journal of Human Virology*, 2, 261: 1999).

Our recent studies provided evidence that HIV protease inhibitors, such as Ritonavir, directly affect cellular proteases, immune cell activation and susceptibility to apoptosis, independent from HIV protease inhibition.

In extension of our previous studies, we measured the effects of Ritonavir on primary endothelial cells (HUVEC) and KS cell lines (KS-Y1 and KSIMM) and found dose-dependent and reversible inhibition of cell proliferation. Drug-effects on induced apoptosis were dependent on the stage of activation and suggested a relation to cell cycling. Activation of NFkB in endothelial cells by TNF, the HIV Tat protein or HHV8 orf 74 was suppressed by Ritonavir in a dose-dependent manner as assessed in luciferase-reporter transfection assays (Figure 15).

Flow cytometry revealed decreases in the expression of NFkB-dependent adhesion molecules VCAM-1, ICAM-1, and E-Selectin in HUVEC and KS cells

that correlated to a functional decrease of monocyte and T-cell adhesion to endothelial cells treated with Ritonavir. NFkB dependent inflammatory cytokine production of IL-6, IL-8, and TNF was also suppressed (Figures 15, 23 and 28).

Since pharmacological interference with apoptosis may favor tumor cell growth and could result in the exacerbation of AIDS related malignancies, we tested the effects of Ritonavir on tumor formation *in vivo*. Application of Ritonavir at doses equivalent to human treatments significantly decreased the formation of subcutaneously implanted tumors in mice (Figures 25-27).

Tumor formation by Kaposi sarcoma (KS) derived cells (using cell lines KSImm and KS-Y1) and leukemia-derived cells (cell lines K562, U937 and KG1a) was similarly inhibited in immune deficient BNX-mice and in immune competent BALB\c mice (Figures 27a-b), indicating that the anti-neoplastic effect of the drug is independent of tumor-specific immune responses. Thus, our *in vivo* model for malignancies and cancer allowed us to substantiate previous anecdotal reports of KS regression in spite of virologic failure (i.e. insignificant decrease in HIV load) under HAART (highly active antiretroviral therapy). It also supports the notion that several mechanisms of tumor suppression may be in effect, including those that inhibit neovascularization, those that enhance tumor cell apoptosis, and those that enhance specific immune responses that leads to elimination of tumor cells as induced by protease inhibitors.

DISCUSSION

The therapeutic application of HIV protease inhibitors (PR-I) commonly leads to substantial increases in CD4+ cell counts but, intriguingly, not always to an immediate decrease, or to only insignificant decreases, in HIV viremia (Levitz et al., *New England J. Med.*, 338/15, 1074, 1998). Even after the initial decrease of plasma HIV load, a viral "break-through" is frequently observed while T-cell counts remain stable and increased (as compared to the time of therapy-initiation). This phenomenon is in discordance to effects usually seen

with other groups of anti-HIV drugs, where development of drug resistance and increase in HIV plasma levels (i.e. increase in viral "load", viral rebound) parallels with significant decreases of T-cell counts (Lederman, S, personal communication 01-1998). Thus, it appears that HIV-PR-I may have additional beneficial effects on T-cell homeostasis.

Marked changes in T-cell subset distribution and lymphocyte function were observed including increases in "memory" CD4CD45RO+ cells and in "naïve" CD4CD45RA+ cells after initiation of PR-I therapy. Reduced expression of activation markers on immune cells such as CD38, CD69 and CD95 as well as restored *in vitro* proliferative responses to mitogens and recall antigens correlate with the clinical improvement of patients that are compliant with PR-I treatment regimens (Kelleher et al., *Journal of Infectious Diseases*, 173:321-329, 1996). While the time course of these changes (when they occur and when they become detectable) depends on the methods and specific criteria, as well as the specific PR-I used and are therefore debatable, the overall message is clear: HIV protease inhibitors, although specifically designed to block HIV protease activity, have additional and direct effects on immune cell functions. These "side effects" likely represent an unappreciated and important part of HIV PR-I's therapeutic effects.

The above-discussed clinical observations motivated us to effect preliminary *in vitro* and *in vivo* investigations utilizing HIV-free cell culture systems and animal models. We surprisingly found that the HIV-PR-I Ritonavir, for example, applied at therapeutic or lower concentrations, increased the colony forming capacity (CFC) of human bone marrow derived mononuclear cells (BM-MNC) in standard methylcellulose cultures up to 160% of that in untreated controls. Peripheral blood derived T-cells responded with phenotypic changes in cell surface marker expression including CD25, CD28, and CD95(Fas)-ligand, as well as with changes in susceptibility to CD95

mediated apoptosis. In addition, treatment of BALB\c mice with HIV-PR-I for three weeks led to a significant increase in peripheral blood white blood cell counts. These results support the idea that HIV-PR-I have, independent from direct HIV blocking, immune enhancing properties.

It is commonly believed that the degree of reduction of apoptosis observed during highly active anti-retroviral therapy (HAART) correlates with decreased levels of HIV replication and that molecular mechanisms controlling susceptibility of T-cells to apoptosis are dependent on the level of HIV replication (or HIV gene product generation). We have demonstrated in this application, for the first time, that the HIV-PR-I Rtionavir improves cell viability/survival in HIV-free *in vitro* systems. The mode of action is independent of HIV infection, since these effects were seen in activated T-cells derived from normal donors. Our data indicates that TNF/NGF receptor ligands are modulated and cellular proteases are affected by Ritonavir. These effects are most pronounced in PBMC fractions activated *in vitro* under conditions that mimic some of the features of the altered immune "environment" seen in HIV-disease (i.e. TCR signaling in the presence of an abnormal cytokine profile). Our *in vitro* observations provide a basis of explanation as to why changes in CD4+T-cell counts and HIV viral load can be "disconnected" *in vivo*.

In our studies, susceptibility to activation induced cell death and apoptosis was decreased by Ritonavir by a mechanism that included, but was not limited to, effects on Casp3- and CD95-dependent apoptosis pathways. It is not clear at the moment to what degree Casp3- independent cell death is altered and what effects PR-I have on other cytokine production. Our data indicates that production of apoptosis mediators that are ligands for "death receptors", such as CD95-L and TNF, are inhibited as well. Although our study was not designed to delineate different pathways of apoptosis that are altered by

Ritonavir, future research should include assessment of other cellular proteases, as well as members of the bcl-2 protein family, since homeostasis regulation, and its dysregulation in HIV disease, is a rather complex process.

Our assays did not allow distinguishing between primary and secondary effects of Ritonavir in affecting cell death. Currently, we speculate that a cross-reactivity of the HIV-PR-I with cell derived proteases is responsible for alterations in cellular activation and susceptibility to apoptosis. In support of this, broad reactivity and overlapping "specificity" have been reported for cellular cysteine proteases and their inhibitors (Margoline et al., *J. Biol. Chem.*, 272:7223, 1997; Alden et. al., *Toxicol. Pathol.*, 25:113, 1997; Wang et. al., *J. Biol. Chem.*, 273:22490, 1998) and also for viral enzymes (Bottcher et al., *Nucleic Acids Res.*, 25:1709, 1997). Other proteases or their secreted inhibitors, such as those responsible for regulating TNF receptor shedding, have been postulated but not yet identified (Aderka D, *Cytokine Growth Factor Rev.*, 7:231, 1996). Our data suggest that the protease-inhibiting activity of Ritonavir may not be exclusively restricted to HIV protease and that cellular proteases involved in cell activation and death may also be effected by the drug.

Very recently, the possible additional effects of HIV PR-I on immune cells were reported by Andre and coworkers (Andre et. al., *PNAS*, 95:13120, 1998). In that experimental system, Ritonavir inhibited 50% of the chymotrypsin-like activity of the 20S proteasome *in vitro* at concentrations ≥5 μM, but inhibition of proteasome housekeeping functions (as assessed by IkappaB degradation) was only detectable at PR-I concentrations as high as ~100 μM. Since the concentrations necessary to observe the immune modulating effects of Ritonavir in our experiments differ by more than 1000-fold, it is likely that cellular pathways different from those reported previously are affected.

Pharmacological modulation of apoptosis may have an immense therapeutic potential. However, interference with such an essential physiologic process could also result in exacerbation of disease processes that are controlled by apoptosis and thereby lead to a disruption of homeostasis (Ashkenazi et. al., *Science*, 281:1305, 1998). The utility of apoptosis inhibition in HIV disease has been debated, since some *in vitro* studies indicate an increase of HIV replication in the presence of ICE (Casp1) inhibitors (Kobayashi et al., *PNAS*, 87:9620, 1990; Sarin et. al., *J Immunol.*, 153:862, 1994; Chinnaiyan et. al., *Nature Medicine*, 3:333, 1997). It has been suggested that the activation of a cell suicide program may serve as a host defense mechanism against viral replication (Clouston et. al., *Med. Hypotheses*, 18:399, 1985; Clem et. al., *J. Virol.*, 67:3730, 1993). We argue that apoptosis as a functional means of immune surveillance against HIV infected cells is unlikely, since cells that harbor HIV are less susceptible to apoptotic stimuli and actual death is executed by means other than ligand-induced apoptosis pathways (Gandhi et. al, *J. Exp. Med.*, 187:1113, 1998; Kolesnitchenko et. al., *J. Virol.*, 71:9753, 1997). We propose that apoptosis in HIV disease eliminates parts of the potential HIV target cell pool, i.e., highly specific, activated effector cells that are most sensitive to apoptotic stimuli. This is supported by *in vitro* experiments, where induction of CD95 mediated apoptosis limited HIV production in cell cultures by eliminating the infection-susceptible pool of activated cells (Sloand et. al, *Blood*, 89:1357, 1997; Kobayashi et. al., *PNAS*, 87:9620, 1990). Thus, we hypothesize that in the apoptosis-stressed immune system of an HIV-infected individual, Ritonavir enhances T-effector cell (and possibly progenitor cell) survival, resulting in increases in cell numbers and a more effective immune response. Recent data (published by Hellerstein and coworkers (Hellerstein et. al., *Nature Medicine*, 5-1:83, 1999)) regarding kinetics of circulating T-cells in HIV-infected individuals support our *in vitro*

finding by extending its relevance to T-cell production and immune progenitor cell activation. Others and we have previously reported that increased levels of inflammatory cytokines (such as TNF) inhibit hematopoiesis *in vitro* by mechanisms that include apoptosis of bone marrow derived progenitor cells (Maciejewski et. al., *J. Immunol.*, 153:4303, 1994).

Our data might supports a conclusion that the greatest clinical success achieved with any anti-HIV drug thus far could be due to unintended side effects on immune cell activation. Assessment of the basic mechanism of clinical improvement and partial restoration of immune functions will lead to new therapeutic strategies, and also have ramifications for the development of vaccines. Consideration of ways to regulate inflammatory responses are important for future vaccine research, and "systemic adjuvants" like Ritonavir could beneficially effect the "set point" of an immune system for immunization, and other similarly acting protease inhibitors. Further characterization of the phenomenon we describe here may provide important clues about HIV mediated pathogenesis. The possibility of restoration of immune function in HIV infected individuals by a controlled prevention of apoptosis deserves more attention, especially now that apoptosis levels are being used as a marker for HIV disease progression.

All references cited herein are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method to modulate the immune system of a host comprising administering a therapeutically effective amount of a protease inhibitor.

2. The method of Claim 1, wherein said protease inhibitor is an HIV protease or proteasome inhibitor, serine protease, or cysteine protease inhibitor.

3. The method of Claim 2, wherein said HIV protease inhibitor is selected from the group consisting of Ritonavir, Saquinavir, Nelfinavir and Indinavir.

4. The method of Claim 1, wherein said HIV protease inhibitor is Ritonavir.

5. A method of treating a disease or condition of the immune system comprising administering a therapeutically effective amount of a protease inhibitor.

6. The method of Claim 5, wherein such protease inhibitor is an HIV protease, proteasome, cysteine protease, or serine protease inhibitor.

7. The method of Claim 6, wherein said HIV protease inhibitor is selected from the group consisting of Ritonavir, Squinavir, Nelfinavir and Indinavir.

8. The method of Claim 6, wherein said HIV protease inhibitor is Ritonavir.

9. The method of Claim 5, wherein said disease of condition of the immune system is selected from the group consisting of autoimmune diseases, cancers, infectious diseases, allergic disorder, and immune dysregulation.

10. A method to modulate or enhance cellular pathways in a subject in need of such treatment comprising administering an effective amount of a protease inhibitor.

11. The method of Claim 10, wherein said cellular pathways are selected from the group consisting of those that regulate cell proliferation, apoptosis, cell activation, cell survival, cell differentiation, cell maturation, and cell cycle.

12. The method of Claim 10, wherein the method is utilized during cancer therapy.

13. The method of Claim 10, wherein said protease inhibitor is an HIV-1 protease, proteasome inhibitor, serine protease, or cysteine protease inhibitor.

14. A method of treating or inhibiting cancer in a subject in need of such treatment or inhibition by administering an effective amount of at least one protease inhibitor.

15. The method of Claim 14, wherein said protease inhibitor is an HIV-1 protease, proteasome, serine protease, or cysteine protease inhibitor.

16. The method of Claim 10, wherein the treated subject is immuno-compromised.

17. The method of Claim 10, wherein the treated subject has a condition selected from the group consisting of non-HIV-1 infection, wasting syndrome, acute ischemia, and inflammatory condition, trauma, cancer, parasite infection, bacterial infection, fungal infection, shock, trauma, transplantation, and burn.

18. A method for increasing the effectiveness of an anti-cancer treatment selected from the group consisting of chemotherapy, radiation, immunotherapy, cytokine therapy, cell therapy, cell cycle modulator therapy, and gene therapy, by administering an effective amount of at least one protease inhibitor.

19. The method of Claim 18, wherein said HIV protease inhibitor renders cancer cells less neoplastic or more susceptible to apoptosis.

20. The method of Claim 19, wherein said protease inhibitor is an HIV protease, proteasome, serine protease, or cysteine protease inhibitor.

21. A method for modulating cell and tissue maintenance or blood cell homeostasis in a subject in need of such treatment by administering an effective amount of at least one protease inhibitor.

22. The method of Claim 21, wherein said subject is undergoing another therapy that inhibits cell or tissue maintenance or blood cell homeostasis.

23. The method of Claim 21, wherein said protease inhibitor is an HIV protease or proteasome inhibitor.

24. A method for inducing a Th-1 immune response in a subject by administering an effective amount of at least one HIV protease or proteasome inhibitor.

25. A method for enhancing or altering the immunological effects elicited by a vaccine by administering said vaccine in combination with at least one protease inhibitor, wherein said vaccine and protease inhibitor are administered separately, or in combination, and in either order.

26. A method for enhancing tissue or cell regeneration and proliferation during at least one of shock, burn, trauma, post-transplant, by administering an effective amount of at least one protease inhibitor.

27. A method of treating non-HIV-1 viral infection by administering an effective amount of at least one HIV-1 protease inhibitor.

28. The method of Claim 25, wherein said viral infection is caused by a retrovirus.

29. The method of Claim 28, wherein said virus is selected from the group consisting of hepatitis, herpes, influenza, papillomavirus, and HIV-2.

30. A method for protecting cells and tissues of a subject from a cell signal that initiates cell death by administering an effective amount of at least one protease inhibitor.

31. The method of Claim 25, wherein said protease inhibitor is an HIV proteasome, cysteine protease, or serine protease inhibitor.

32. The method of Claim 24, wherein said subject comprises at least one of trauma, inflammation, infection, transplant, and malnutrition.

33. A method for treating an inflammatory condition comprising administering a therapeutically effective amount of an HIV-1 protease or proteasome inhibitor.

34. A method for treating HIV-2 infection by administration of an immunologically effective amount of an HIV-1 protease inhibitor.

FIG. 1

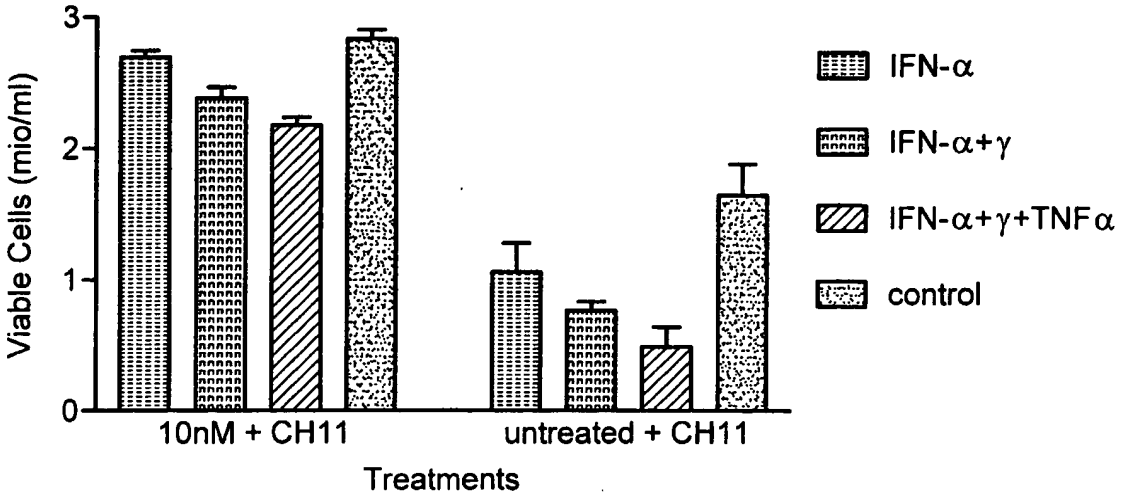

Viable Cells (mio/ml)
3
2
1
0
10nM + CH11
untreated + CH11
Treatments
IFN-α
IFN-α+γ
IFN-α+γ+TNFα
control

FIG. 2

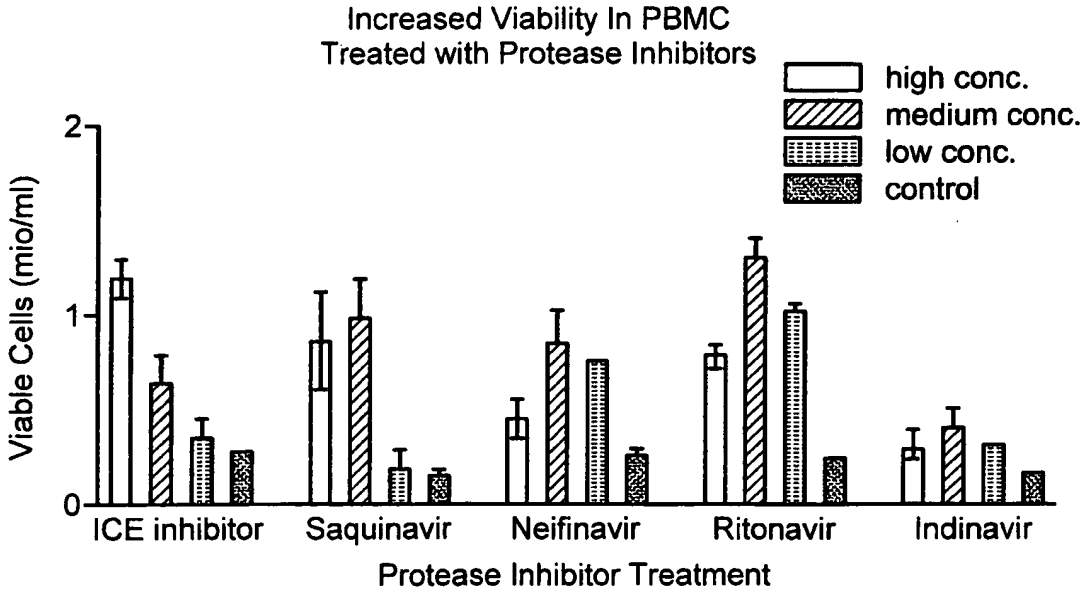

Increased Viability In PBMC
Treated with Protease Inhibitors
Viable Cells (mio/ml)
2
1
0
ICE inhibitor
Saquinavir
Neifinavir
Ritonavir
Indinavir
Protease Inhibitor Treatment
high conc.
medium conc.
low conc.
control

FIG. 3

Decrease of Apoptosis in PBMC
Treated with Different HIV PR-inhibitors

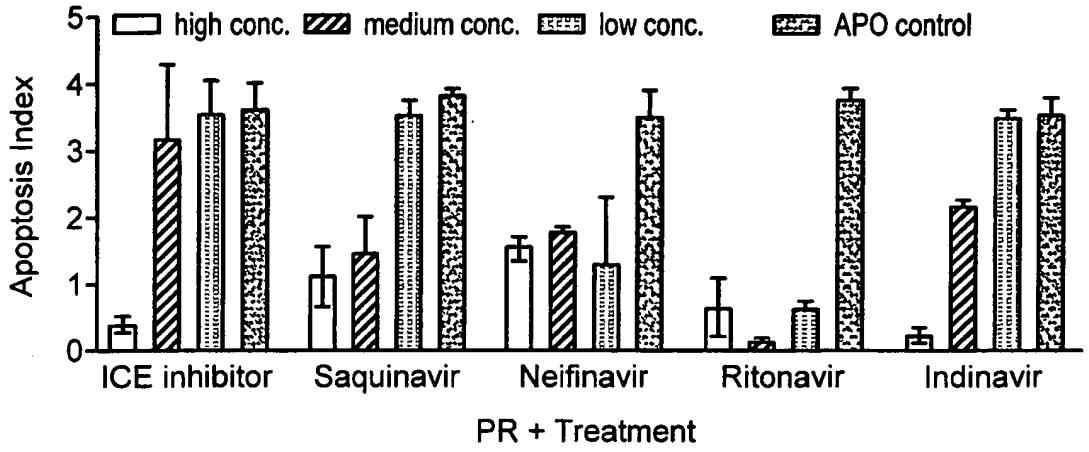
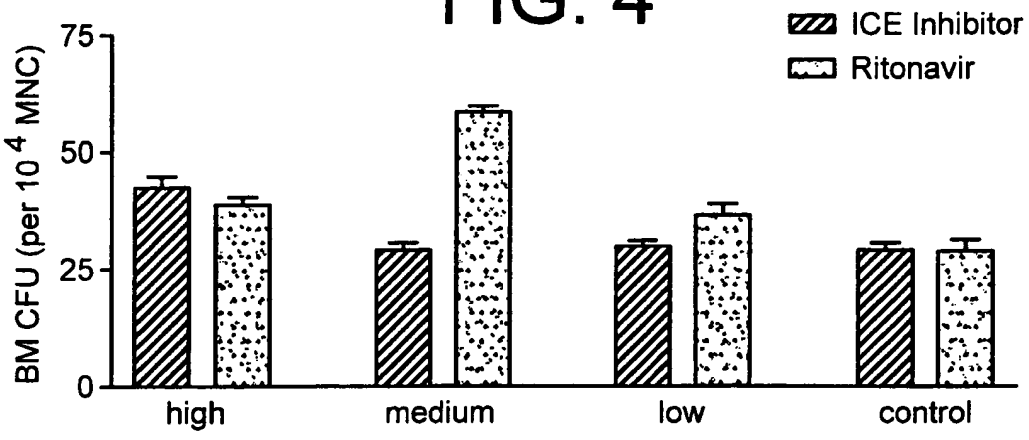


FIG. 4



Caspase 1 (ICE) inhibitor was added to BM cultures at 100 μ M(high), 10 μ M (medium), and 1 μ M (low), Ritonavir was used at 100nM (high), 10nM (medium), and 1nM (low) for treatments; standard methylcellulose culture technique

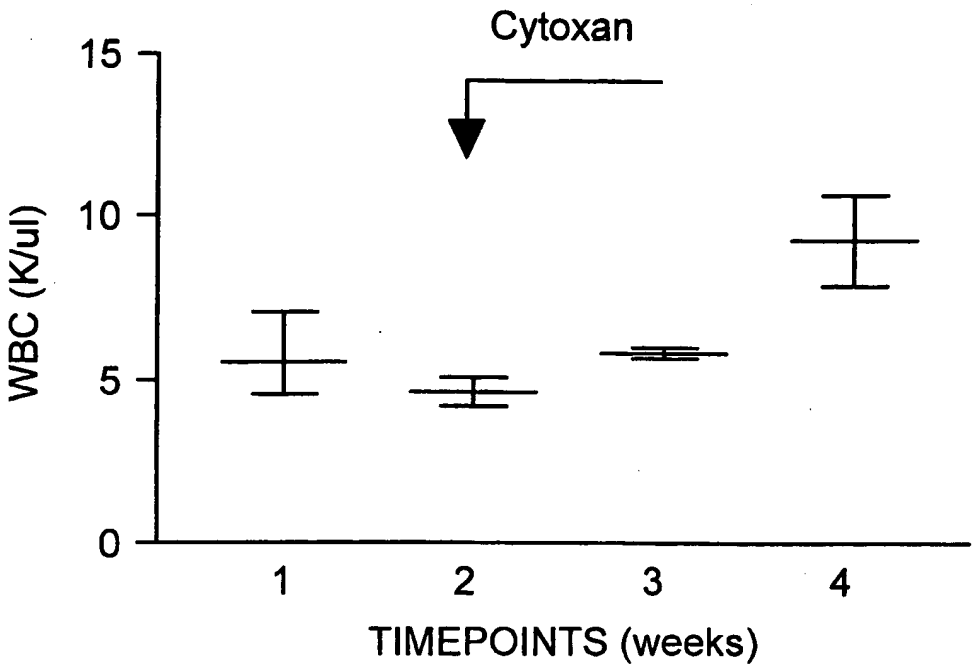

Cytoxan
15
10
5
0
WBC (K/ul)
1
2
3
4
TIMEPOINTS (weeks)

FIG. 5A

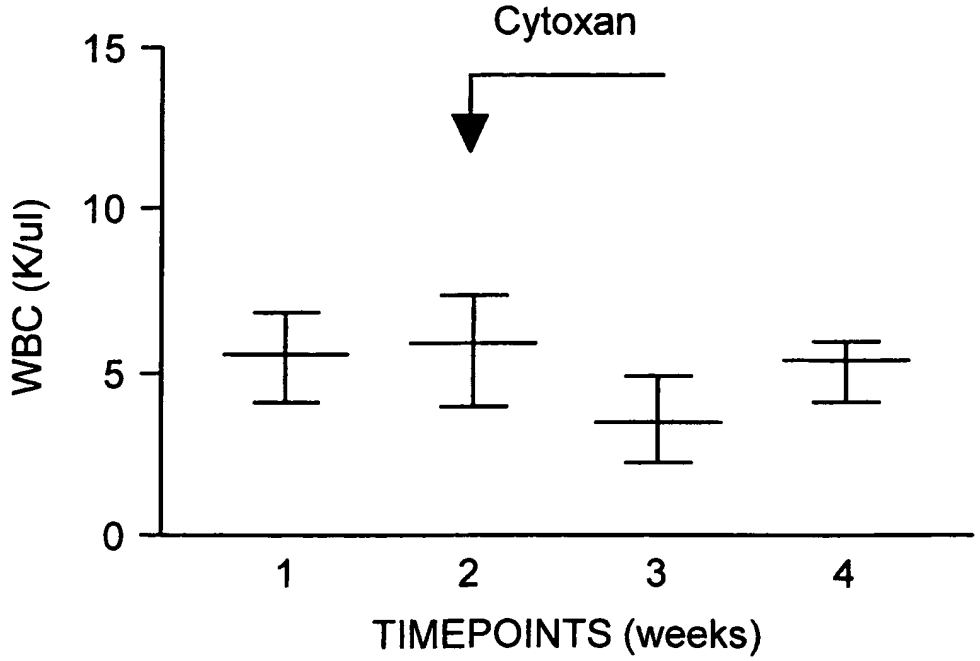

Cytoxan
15
10
5
0
WBC (K/ul)
1
2
3
4
TIMEPOINTS (weeks)

FIG. 5B

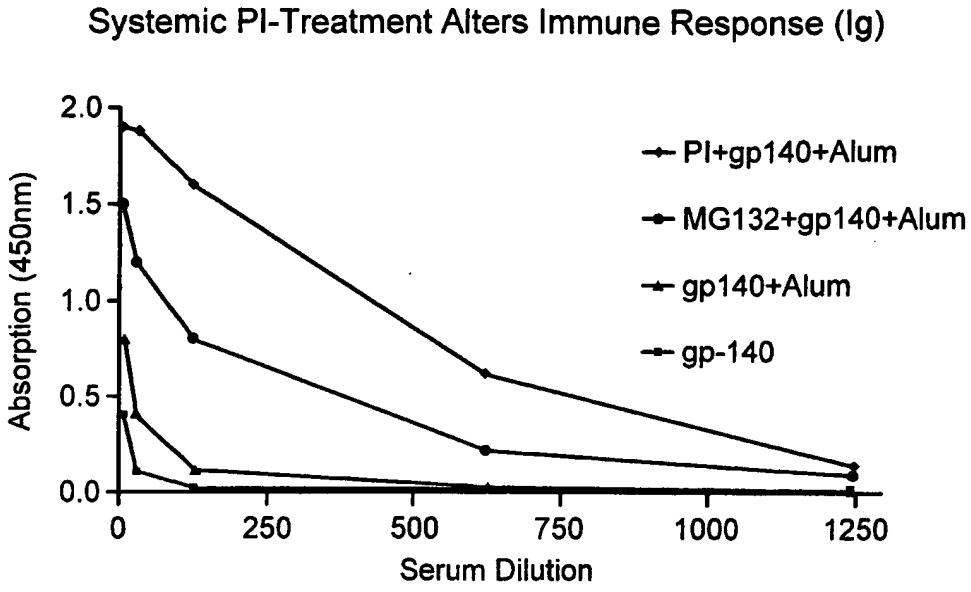

Systemic PI-Treatment Alters Immune Response (Ig)
Absorption (450nm)
2.0
1.5
1.0
0.5
0.0
0
250
500
750
1000
1250
Serum Dilution
PI+gp140+Alum
MG132+gp140+Alum
gp140+Alum
gp-140

FIG. 6A

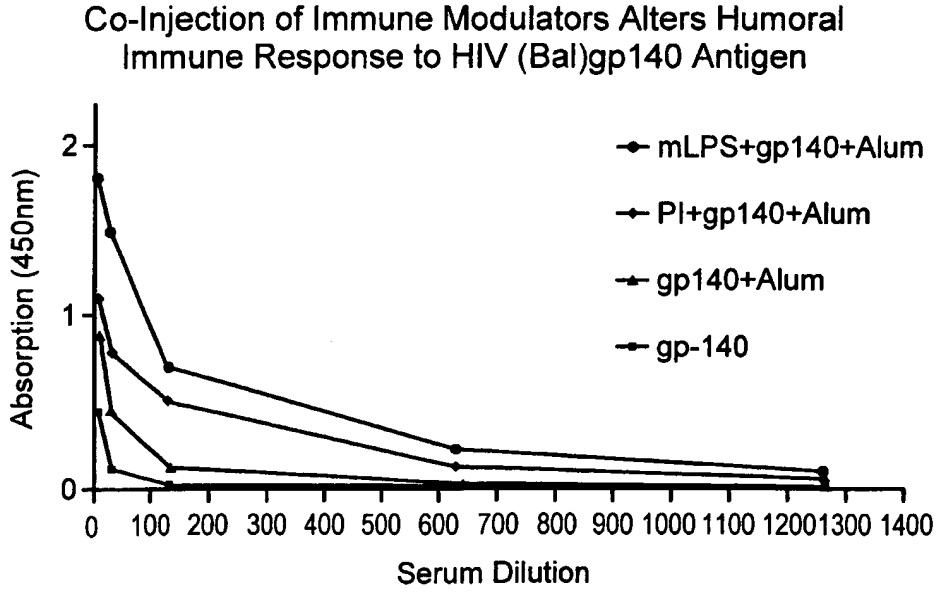

Co-Injection of Immune Modulators Alters Humoral Immune Response to HIV (Bal)gp140 Antigen
Absorption (450nm)
2
1
0
0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400
Serum Dilution
mLPS+gp140+Alum
PI+gp140+Alum
gp140+Alum
gp-140

FIG. 6B

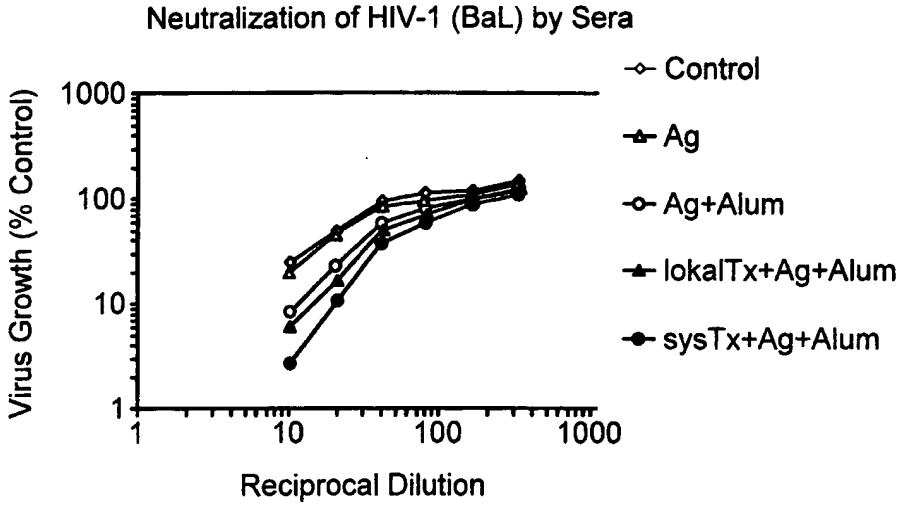

Neutralization of HIV-1 (BaL) by Sera
Virus Growth (% Control)
1000
100
10
1
1
10
100
1000
Reciprocal Dilution
Control
Ag
Ag+Alum
lokalTx+Ag+Alum
sysTx+Ag+Alum

FIG. 6C

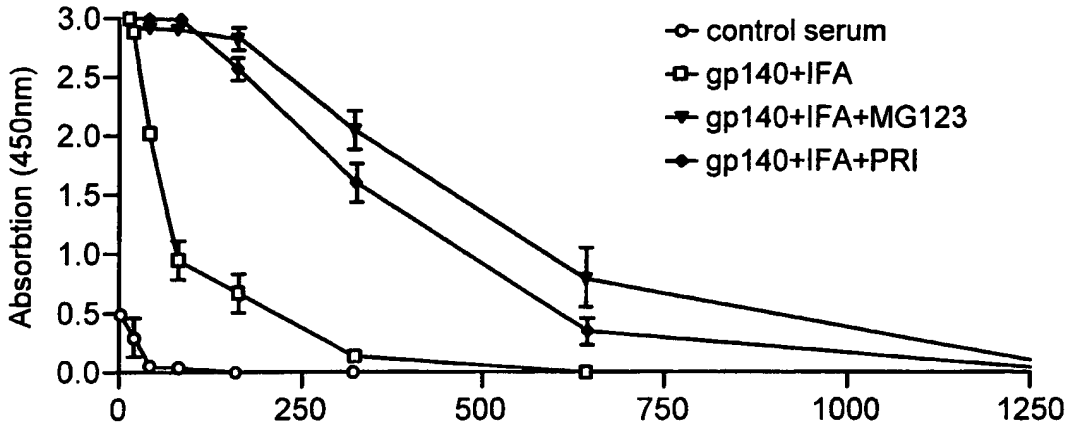

Absorbtion (450nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
250
500
750
1000
1250
control serum
gp140+IFA
gp140+IFA+MG123
gp140+IFA+PRI

FIG. 6D

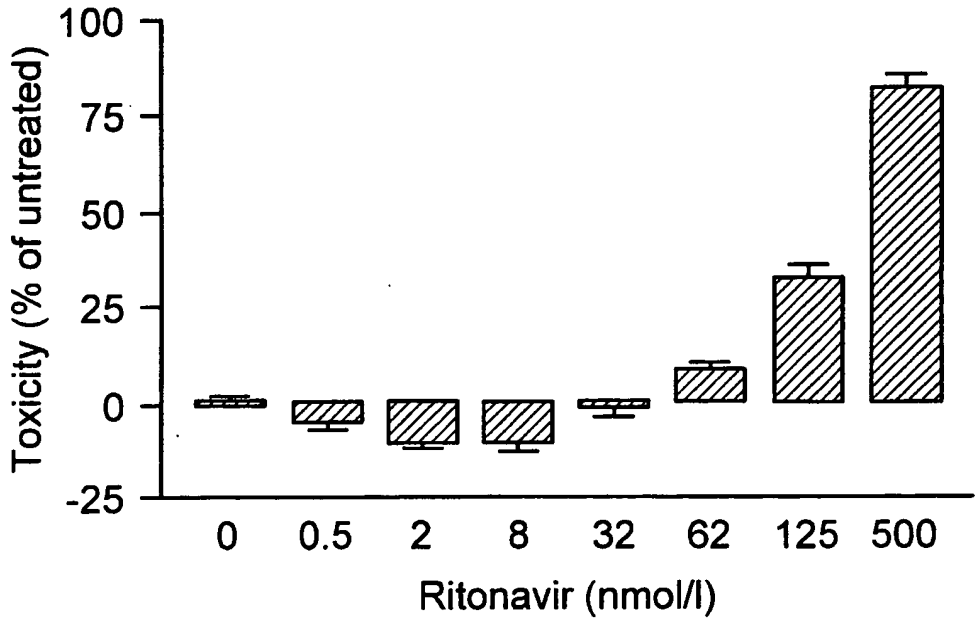

Toxicity (% of untreated)
100
75
50
25
0
-25
0
0.5
2
8
32
62
125
500
Ritonavir (nmol/l)

FIG. 7

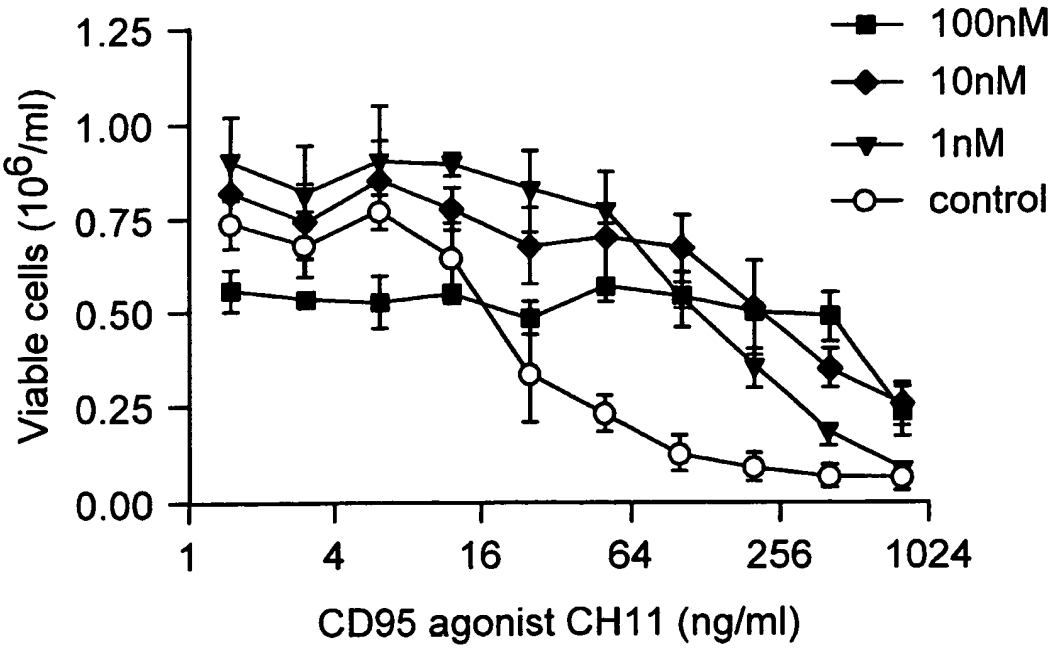

Viable cells (10^6/ml)
1.25
1.00
0.75
0.50
0.25
0.00
1
4
16
64
256
1024
CD95 agonist CH11 (ng/ml)
100nM
10nM
1nM
control

FIG. 9

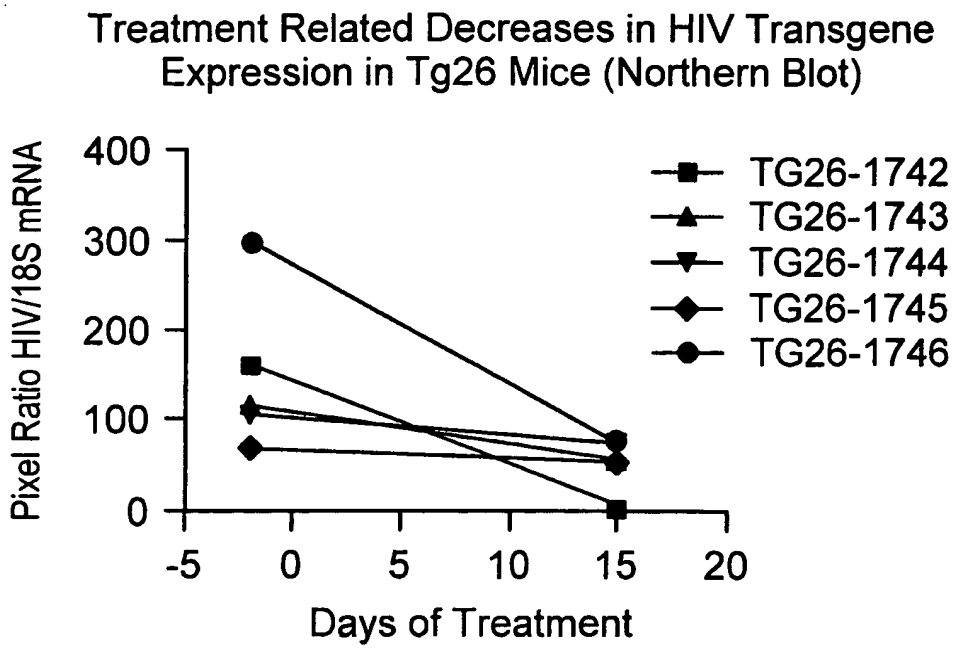

Treatment Related Decreases in HIV Transgene Expression in Tg26 Mice (Northern Blot)
Pixel Ratio HIV/18S mRNA
400
300
200
100
0
-5
0
5
10
15
20
Days of Treatment
TG26-1742
TG26-1743
TG26-1744
TG26-1745
TG26-1746

FIG. 8A

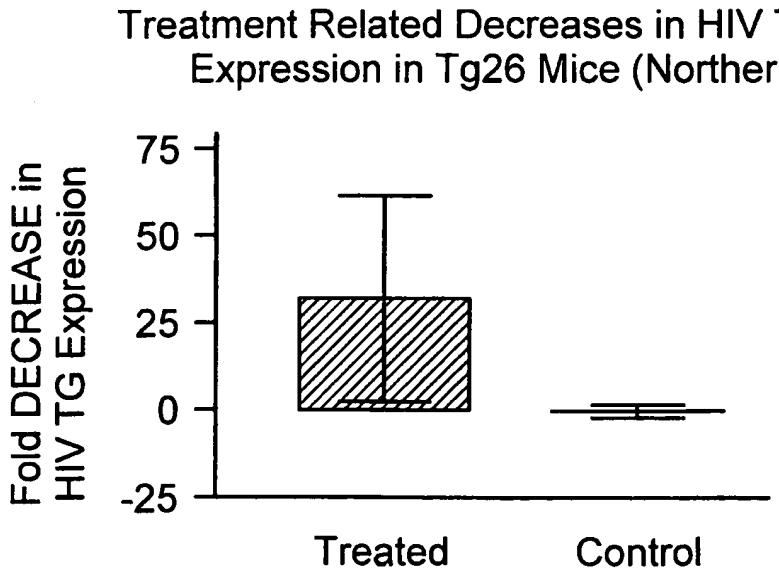

Treatment Related Decreases in HIV Transgene Expression in Tg26 Mice (Northern Blot)
Fold DECREASE in HIV TG Expression
75
50
25
0
-25
Treated
Control

FIG. 8B

FIG. 10A

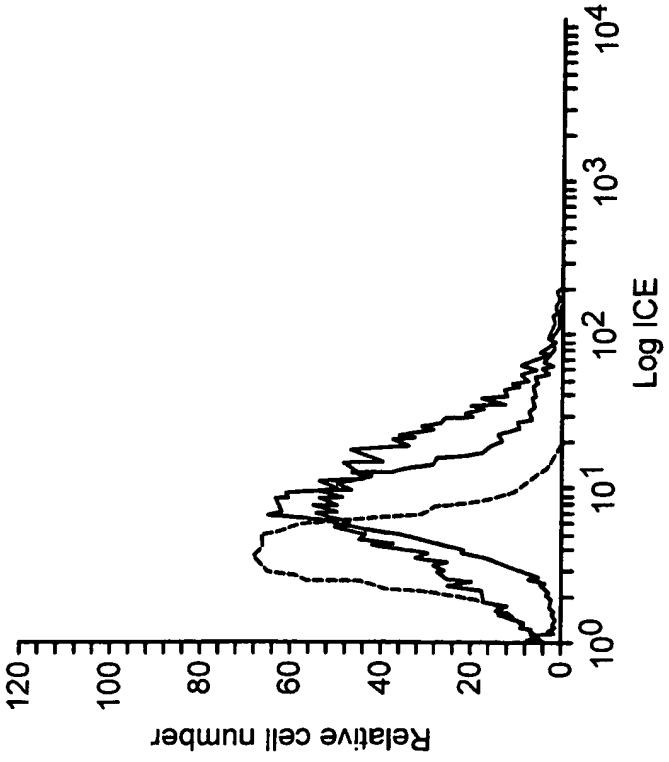


FIG. 10B

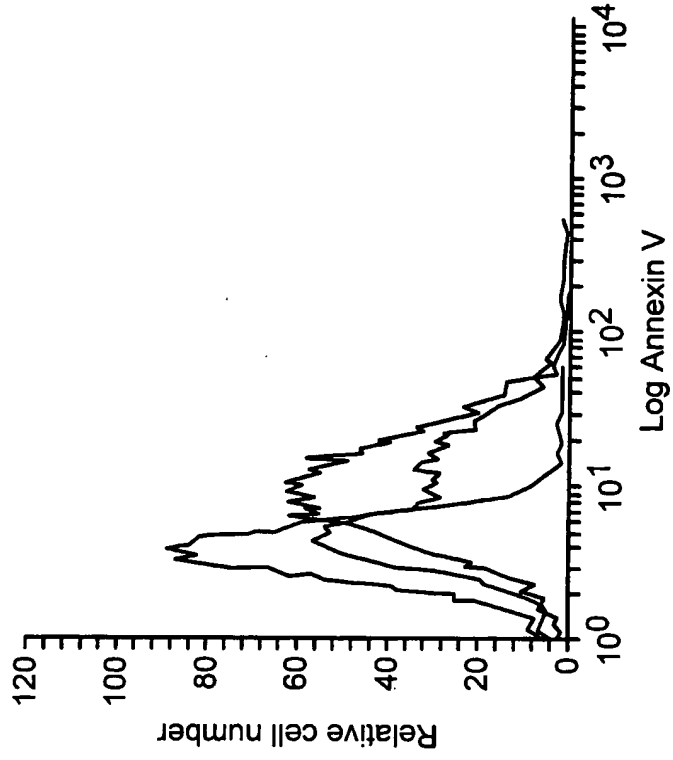
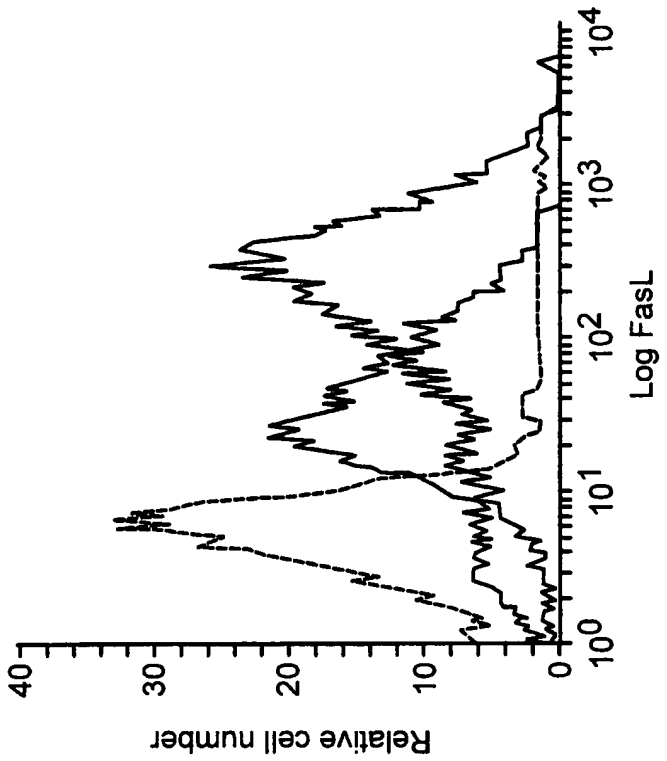


FIG. 10C



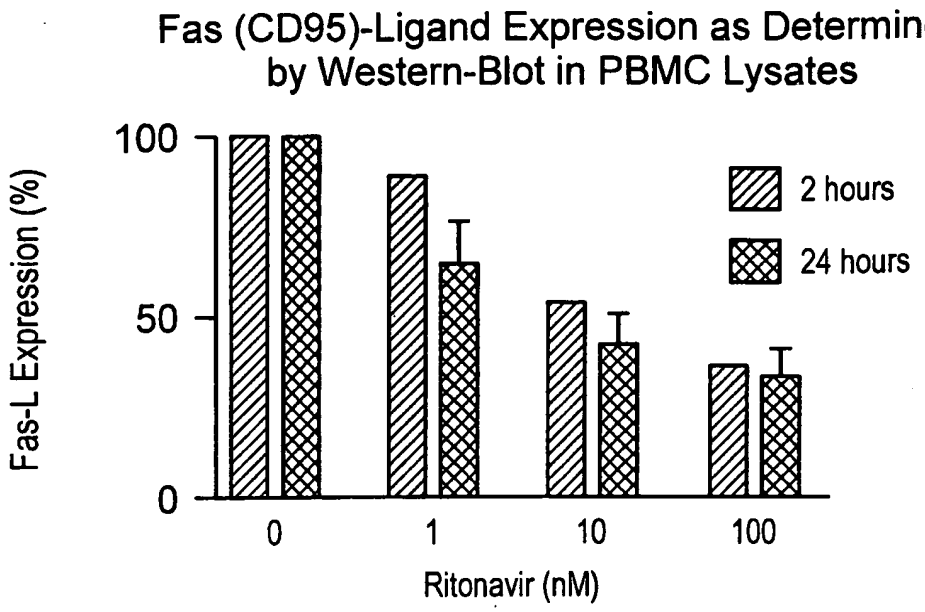


FIG. 10D

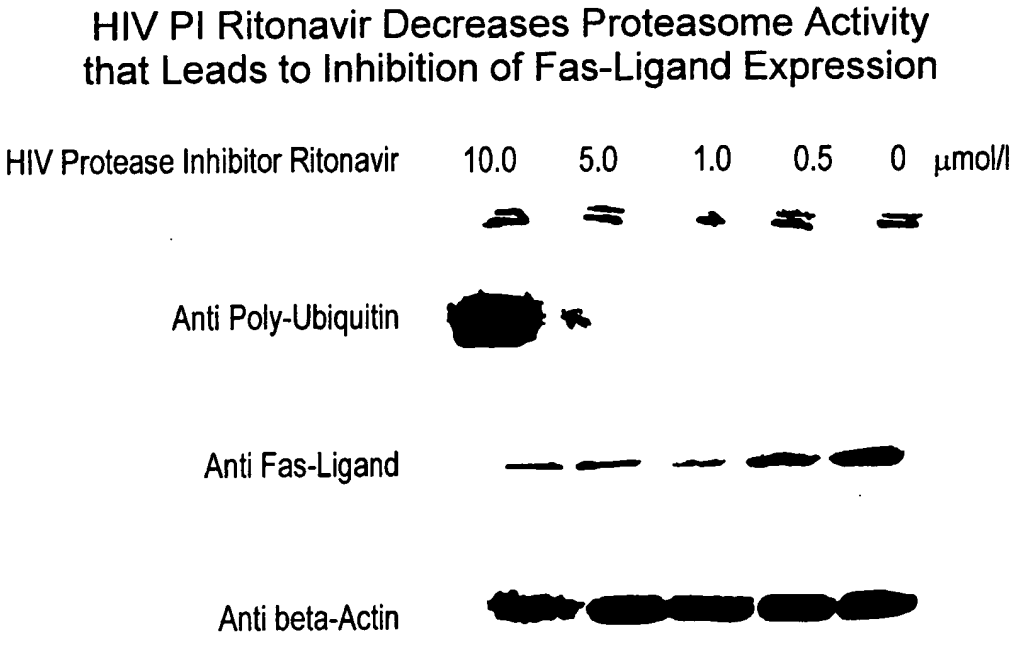


FIG. 10E

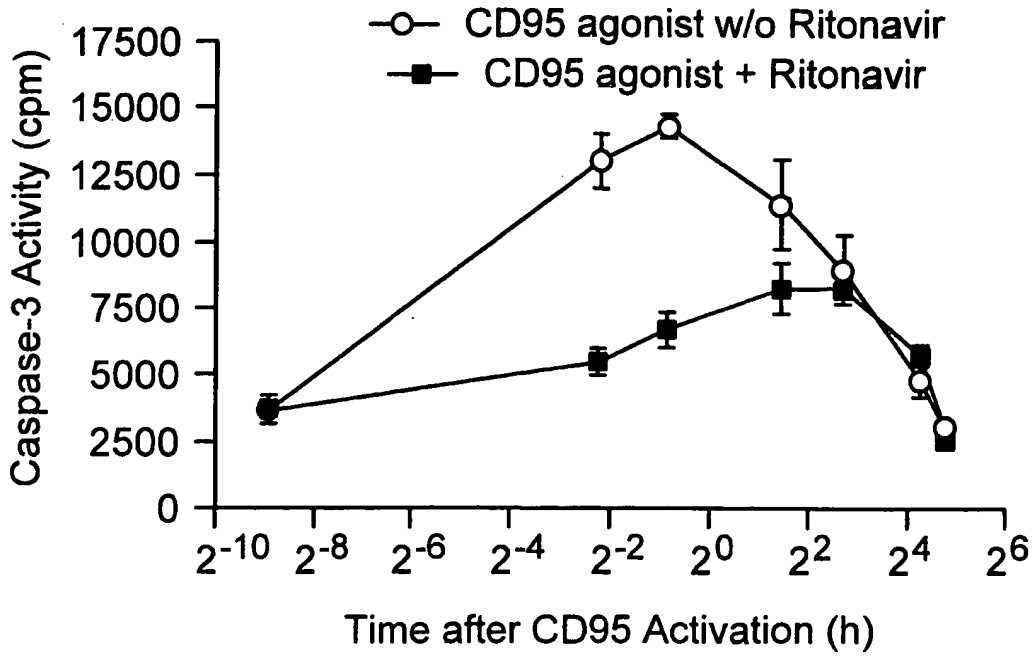


FIG. 11A

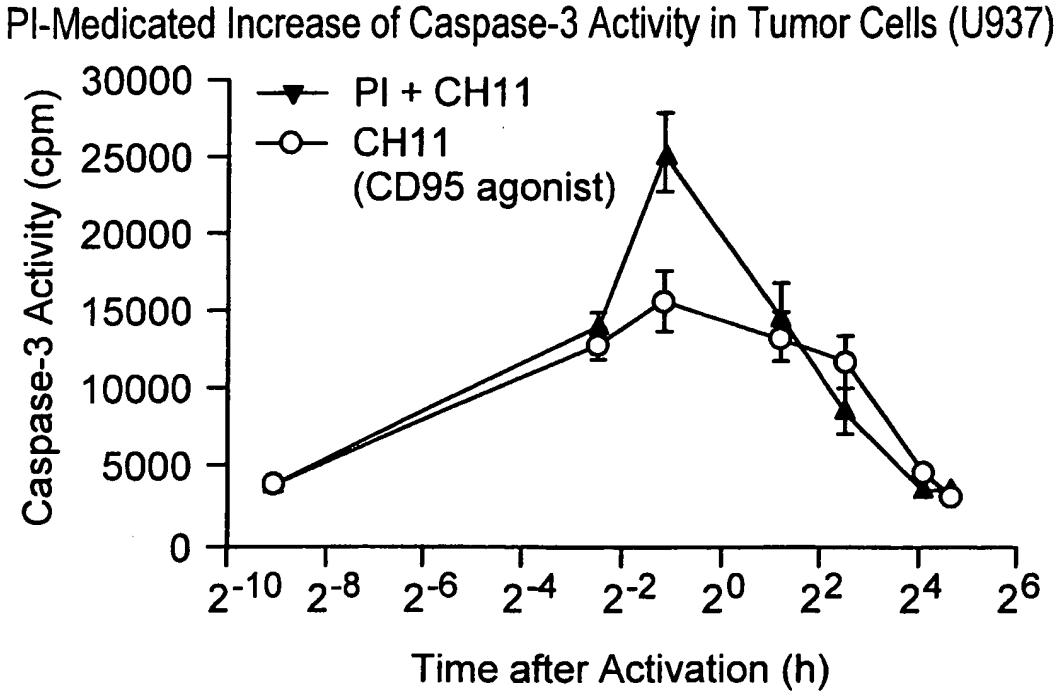
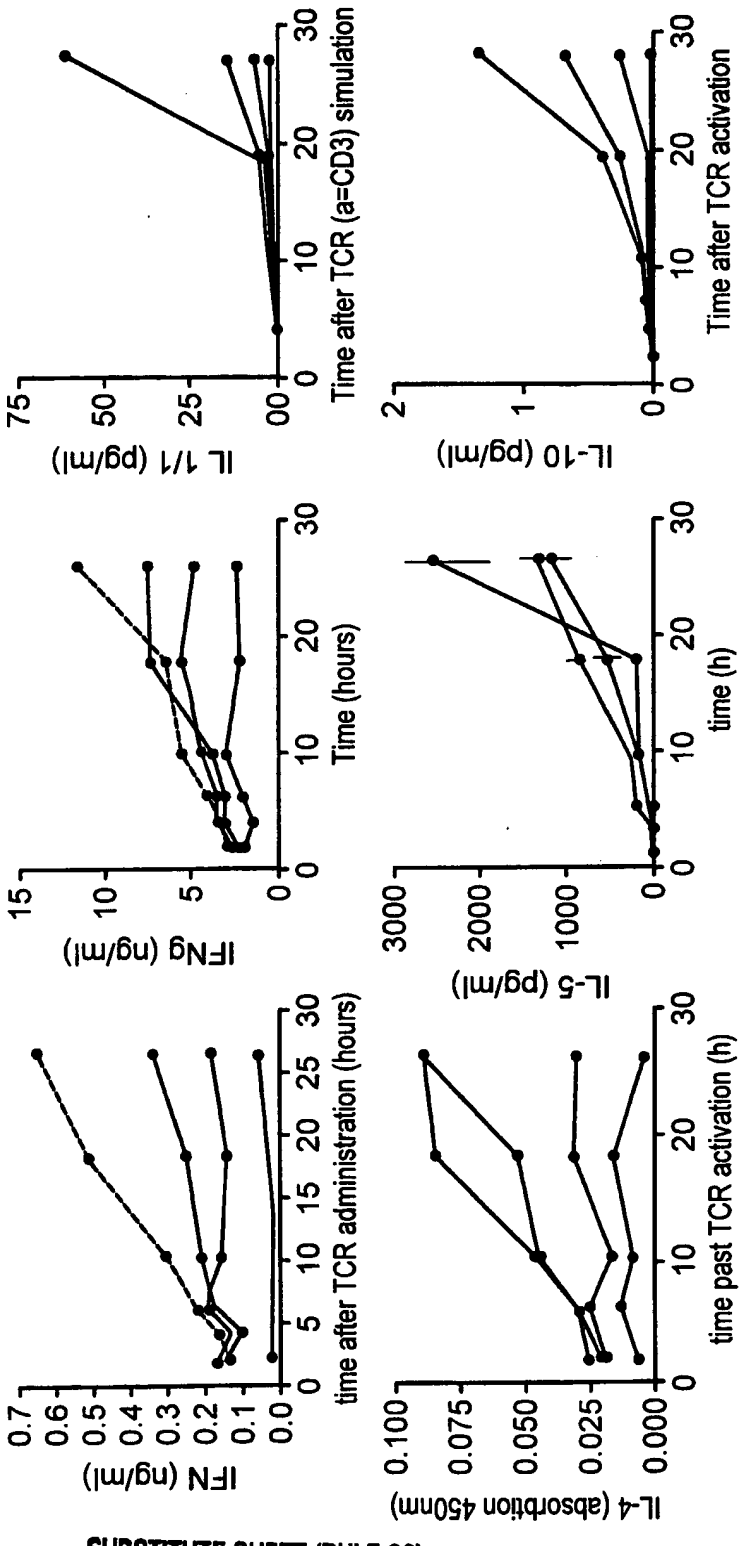


FIG. 11B

FIG. 12

PI Effects on Inflammatory Cytokines



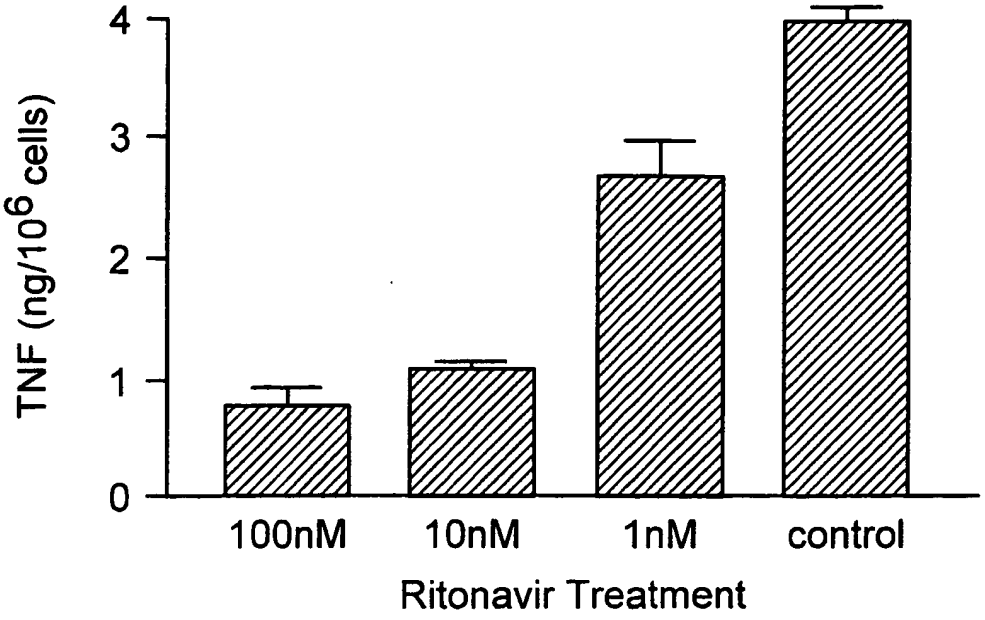

TNF (ng/10^6 cells)
4
3
2
1
0
100nM
10nM
1nM
control
Ritonavir Treatment

FIG. 13

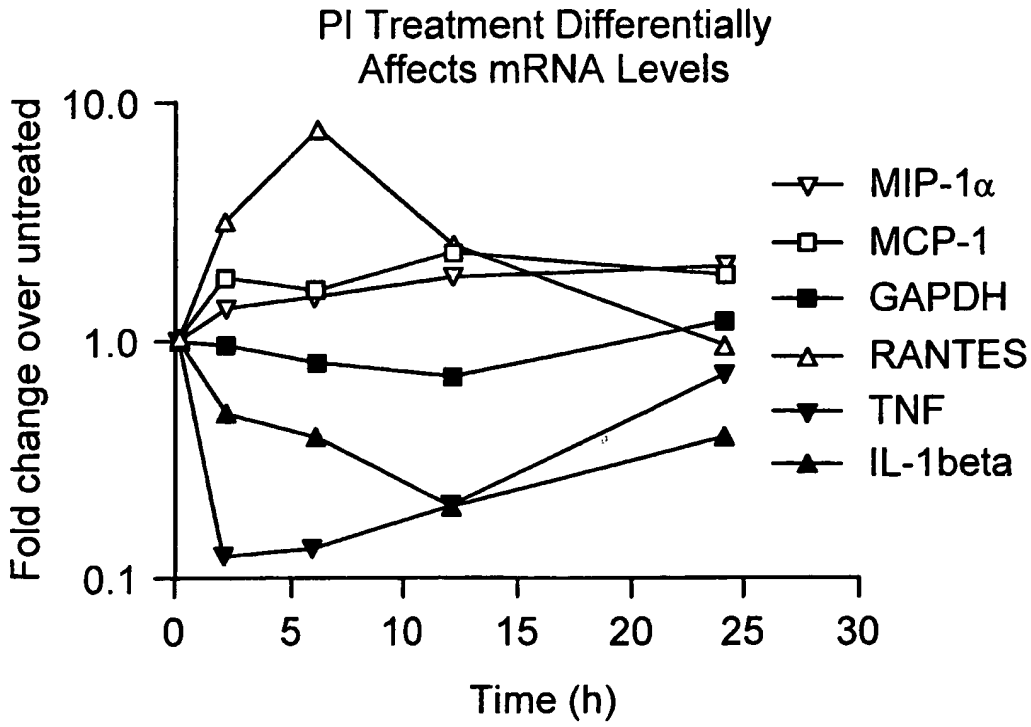

PI Treatment Differentially
Affects mRNA Levels
Fold change over untreated
10.0
1.0
0.1
0
5
10
15
20
25
30
Time (h)
MIP-1α
MCP-1
GAPDH
RANTES
TNF
IL-1beta

FIG. 14

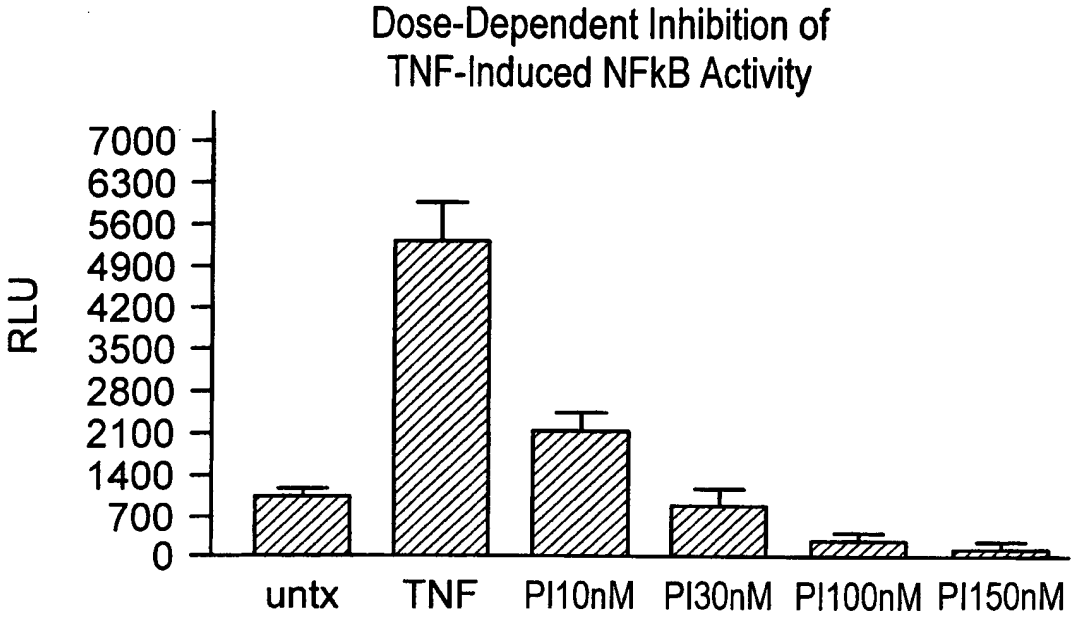

Dose-Dependent Inhibition of
TNF-Induced NFkB Activity
RLU
7000
6300
5600
4900
4200
3500
2800
2100
1400
700
0
untx
TNF
PI10nM
PI30nM
PI100nM
PI150nM

FIG. 15

FIG. 16A

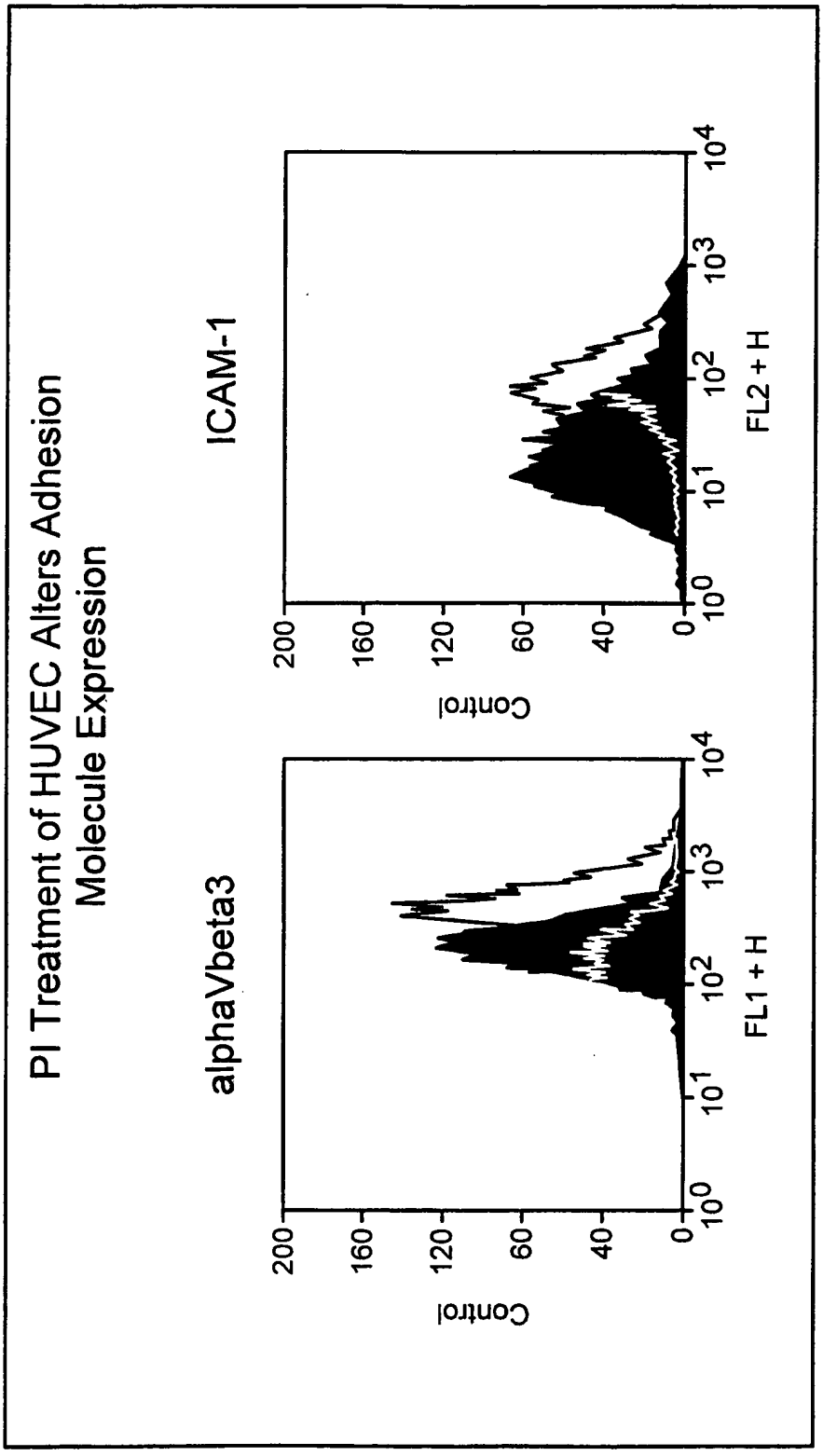

PI Treatment of HUVEC Alters Adhesion Molecule Expression
alphaVbeta3
ICAM-1
Control
FL1 + H
FL2 + H
0
40
60
120
160
200
10^0
10^1
10^2
10^3
10^4

FIG. 16B

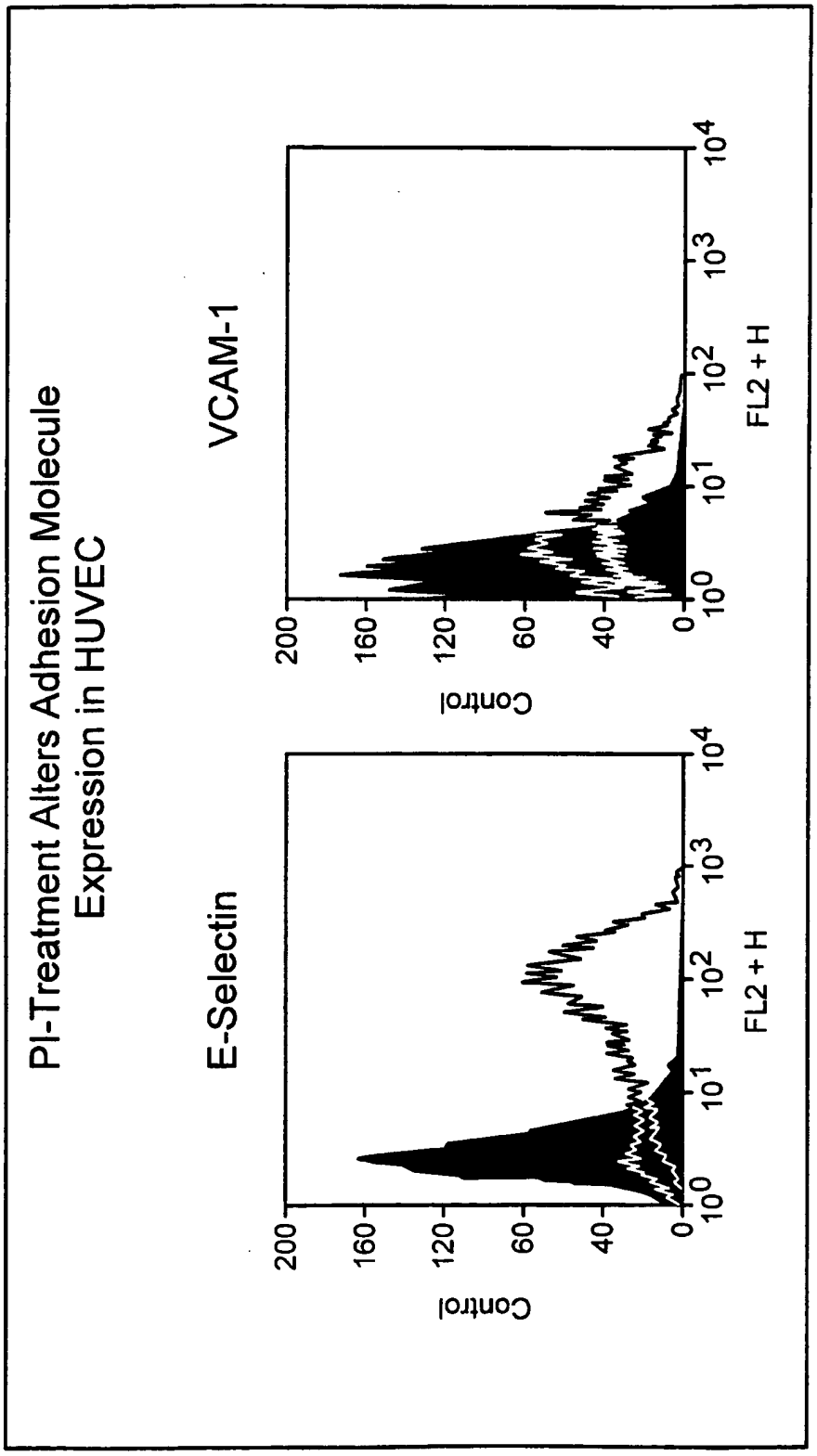
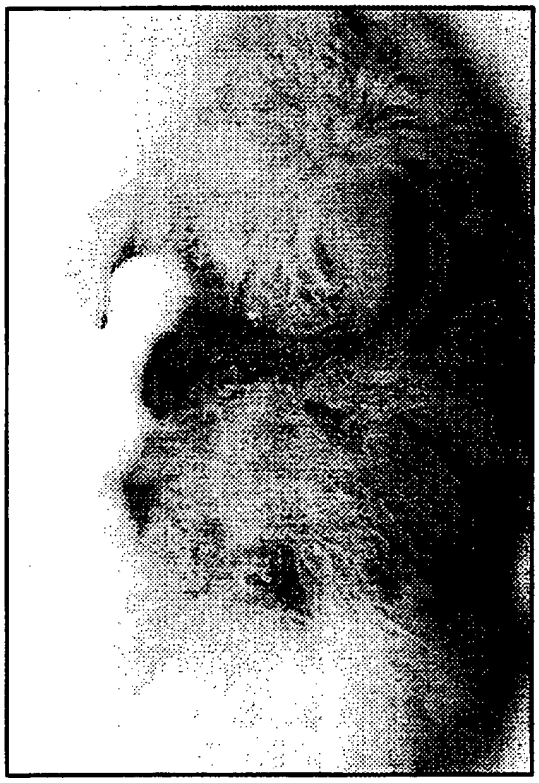

PI-Treatment Alters Adhesion Molecule Expression in HUVEC
E-Selectin
VCAM-1
Control
FL2 + H
0
40
60
120
160
200
10^0
10^1
10^2
10^3
10^4

FIG. 17

Skin Lesions in HIV Transgenic (Tg26) Mice



Two Weeks of Treatment

Before Treatment

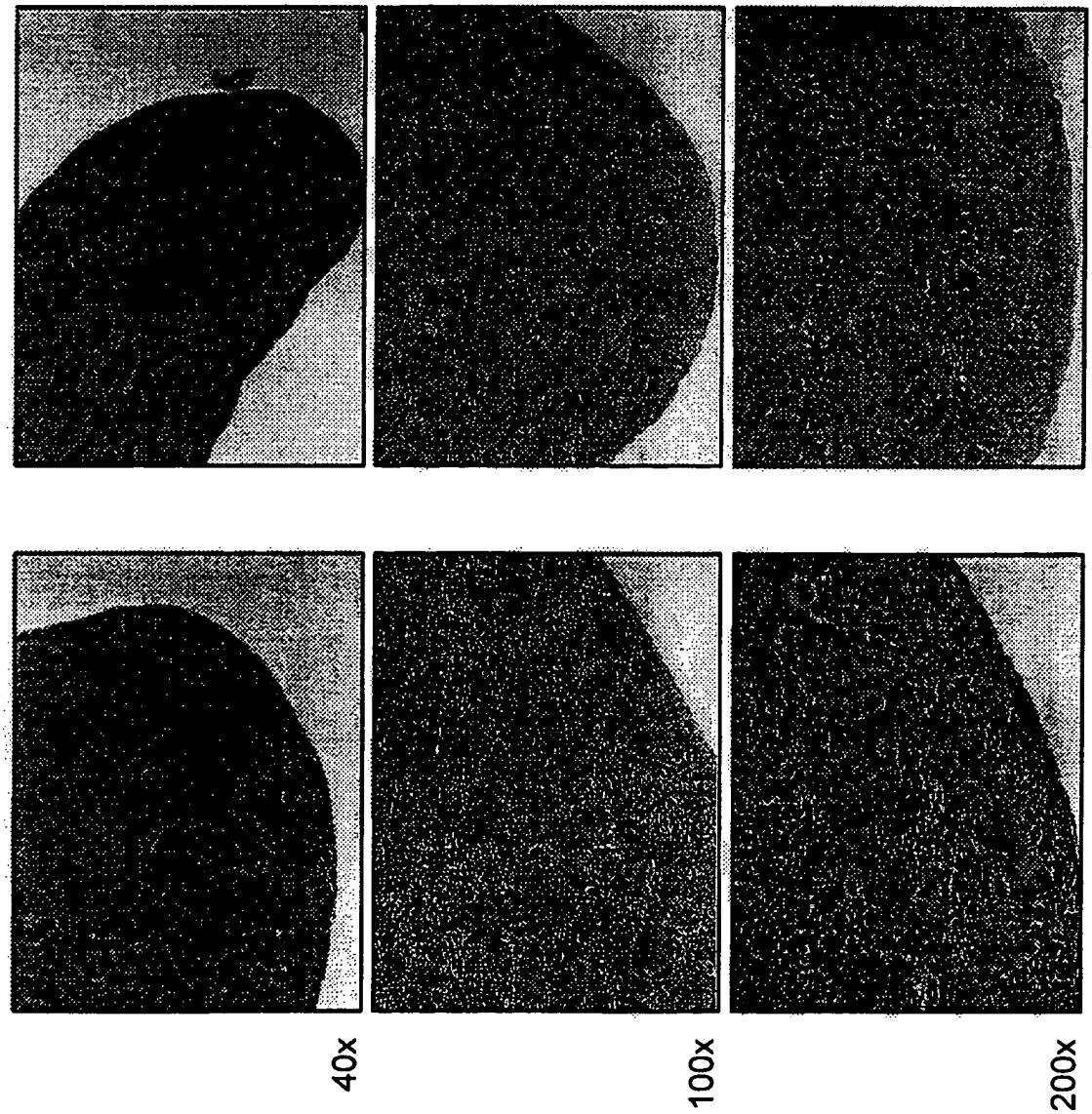


FIG. 18
Spleen
HIV Transgenic
Mouse (Tg26)

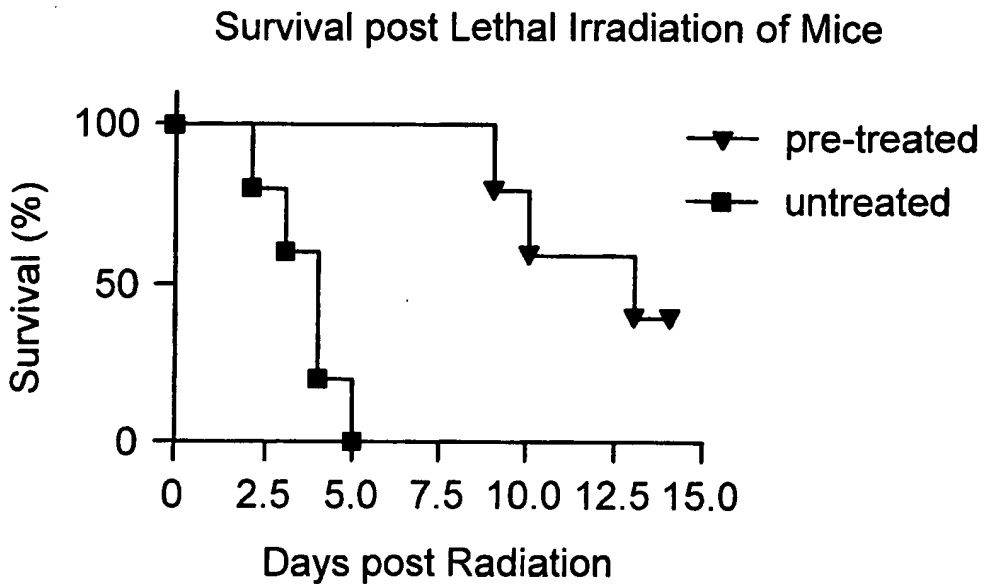


FIG. 19A

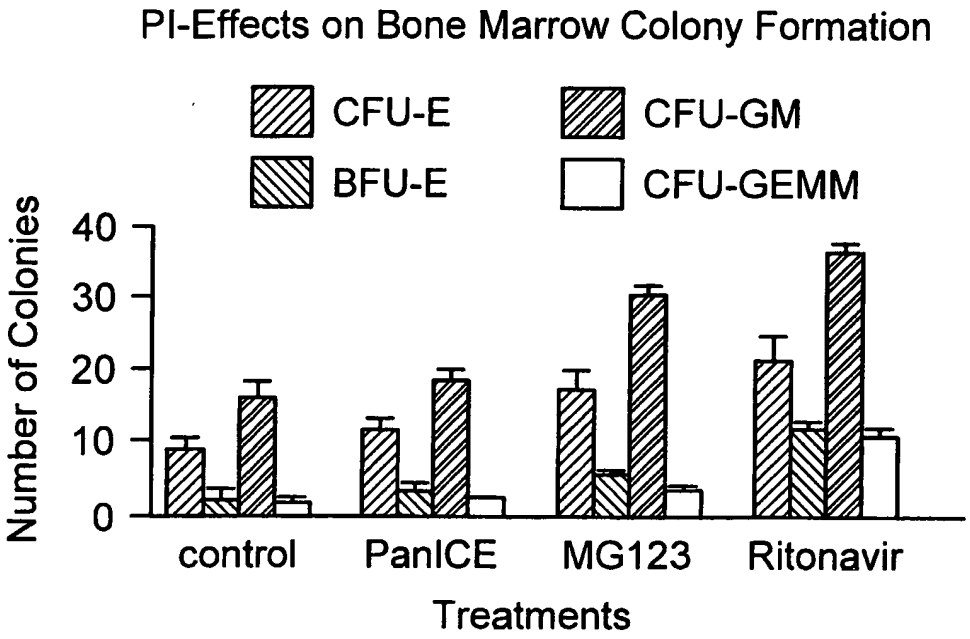


FIG. 19B

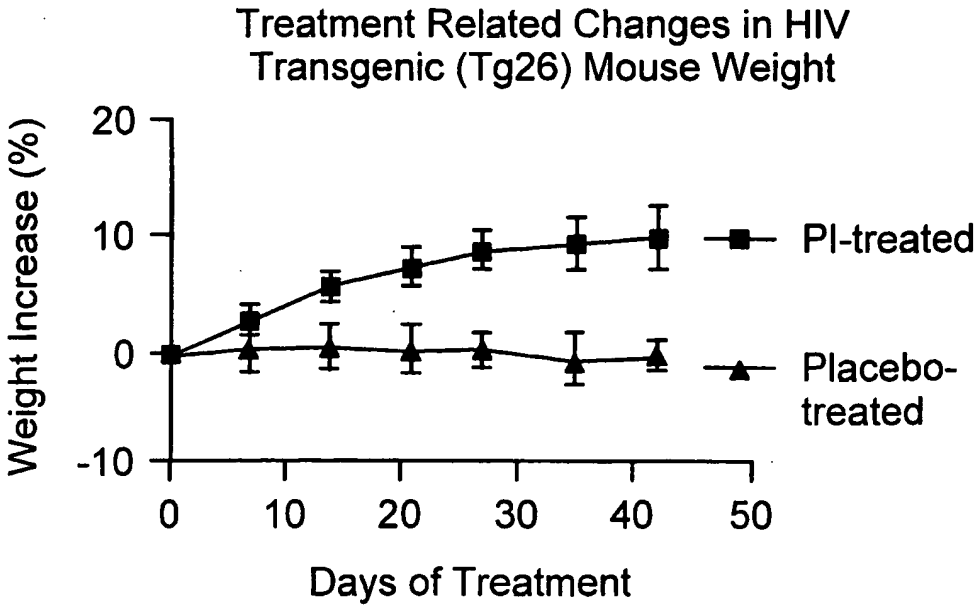

Treatment Related Changes in HIV
Transgenic (Tg26) Mouse Weight
Weight Increase (%)
20
10
0
-10
0
10
20
30
40
50
Days of Treatment
PI-treated
Placebo-
treated

FIG. 20

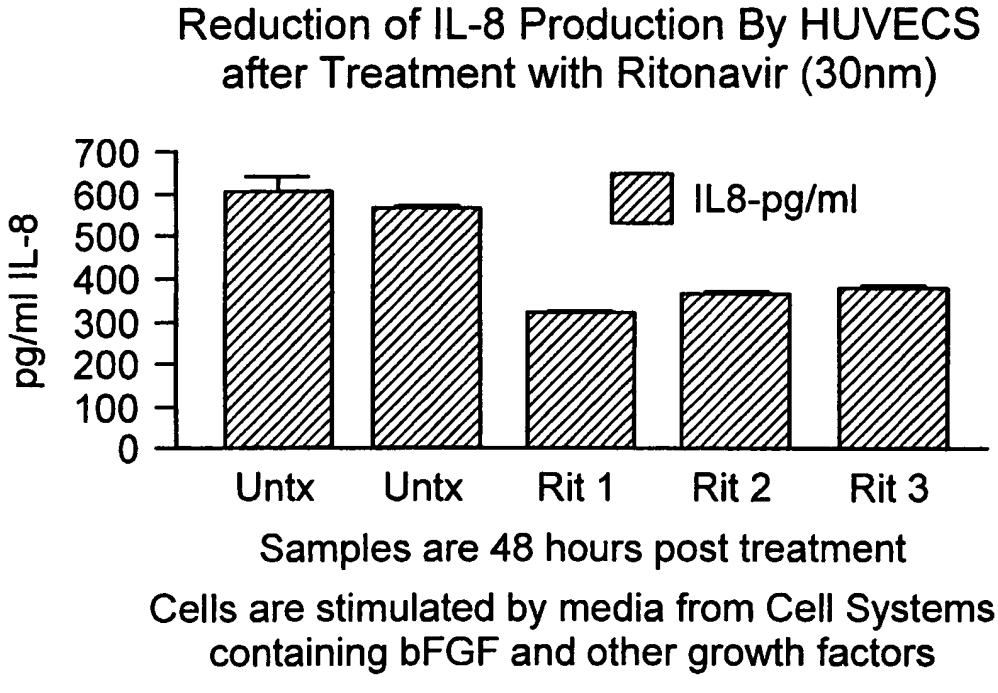

Reduction of IL-8 Production By HUVECS
after Treatment with Ritonavir (30nm)
pg/ml IL-8
700
600
500
400
300
200
100
0
IL8-pg/ml
Untx
Untx
Rit 1
Rit 2
Rit 3
Samples are 48 hours post treatment
Cells are stimulated by media from Cell Systems
containing bFGF and other growth factors

FIG. 21

FIG. 22

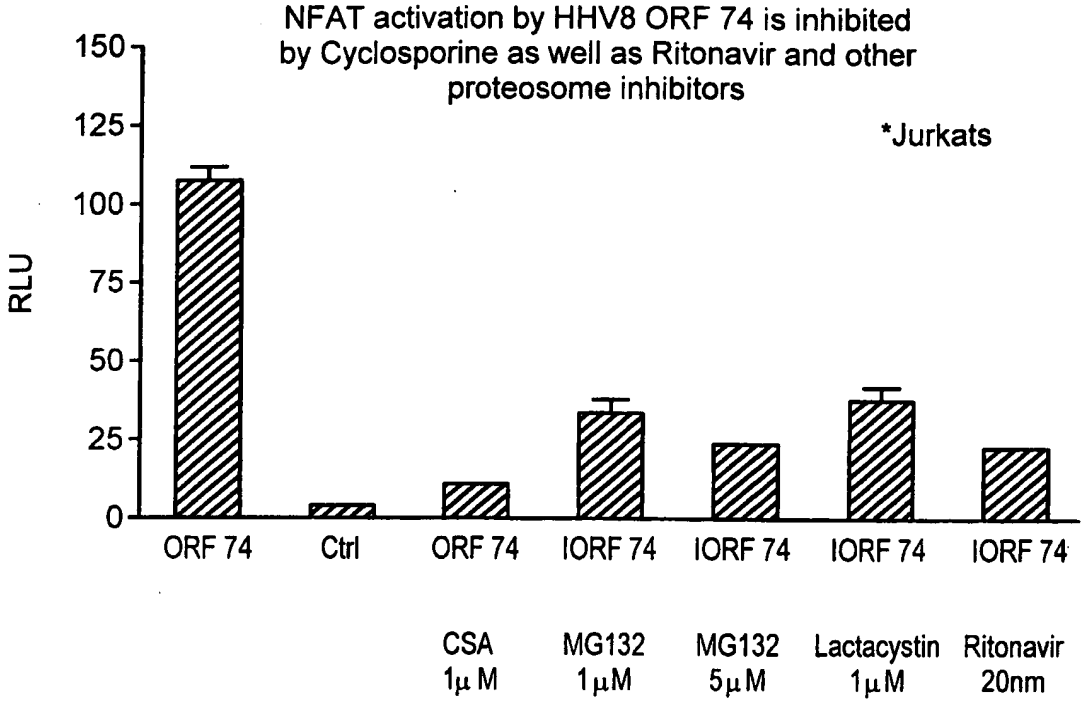

NFAT activation by HHV8 ORF 74 is inhibited by Cyclosporine as well as Ritonavir and other proteosome inhibitors
*Jurkats
RLU
150
125
100
75
50
25
0
ORF 74
Ctrl
ORF 74
IORF 74
IORF 74
IORF 74
IORF 74
CSA 1μM
MG132 1μM
MG132 5μM
Lactacystin 1μM
Ritonavir 20nm

FIG. 23

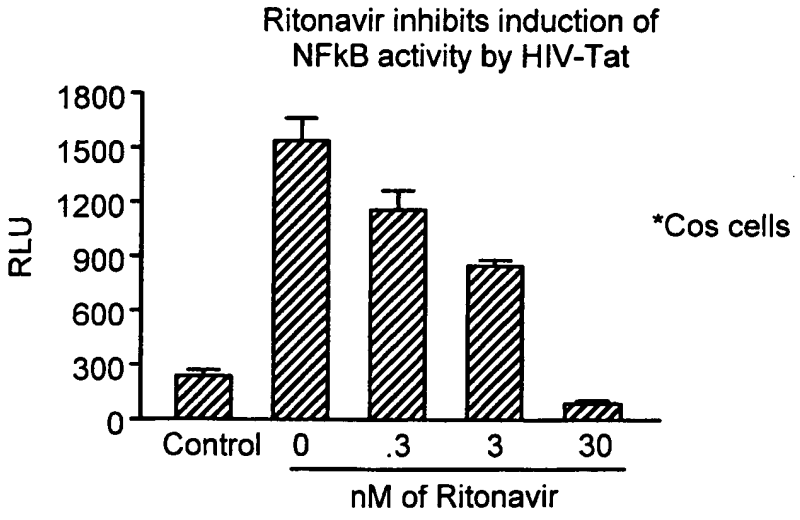
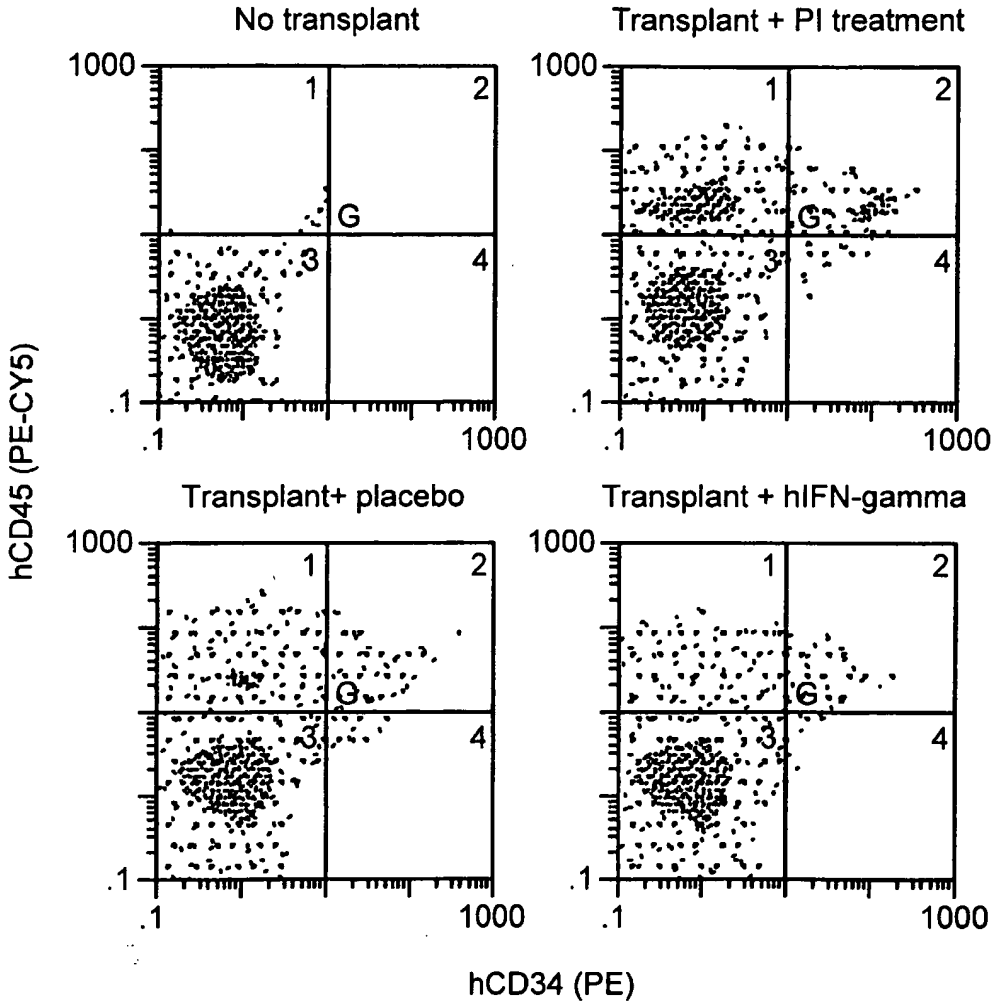

Ritonavir inhibits induction of NFkB activity by HIV-Tat
*Cos cells
RLU
1800
1500
1200
900
600
300
0
Control
0
.3
3
30
nM of Ritonavir

FIG. 24

Engraftment of Human Cord Blood in SCID/NOD Mice



Analysis of SCID/NOD mouse BM after 6 months of transplantation.

FIG. 25A

Inhibition of Tumor Formation by HIV-PI Ritonavir

Beige Nude XID Mice

Untreated control

Treated for two weeks after tumor appearance

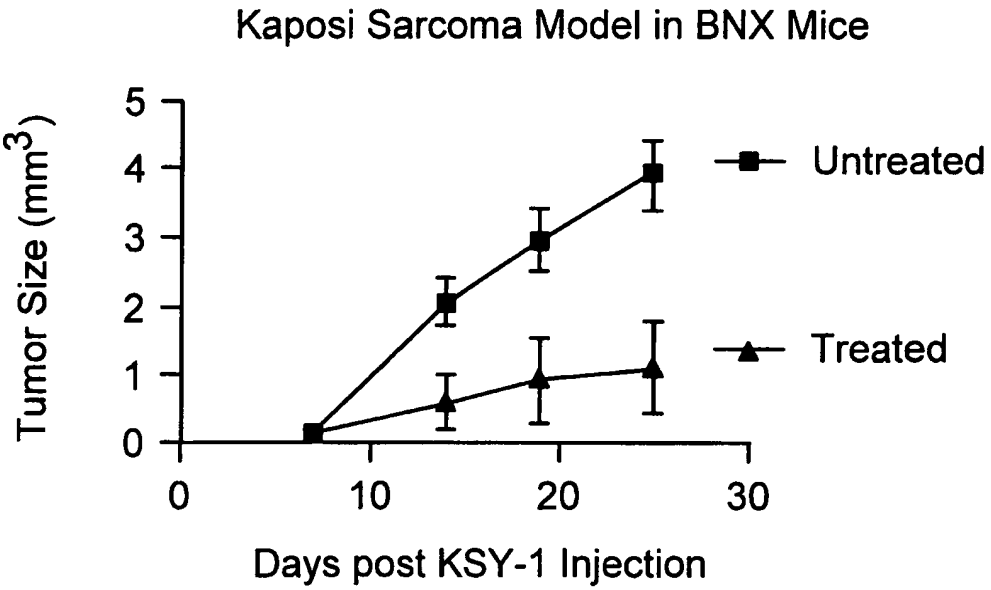

Kaposi Sarcoma Model in BNX Mice
Tumor Size (mm^3)
5
4
3
2
1
0
0
10
20
30
Days post KSY-1 Injection
Untreated
Treated

FIG. 25B

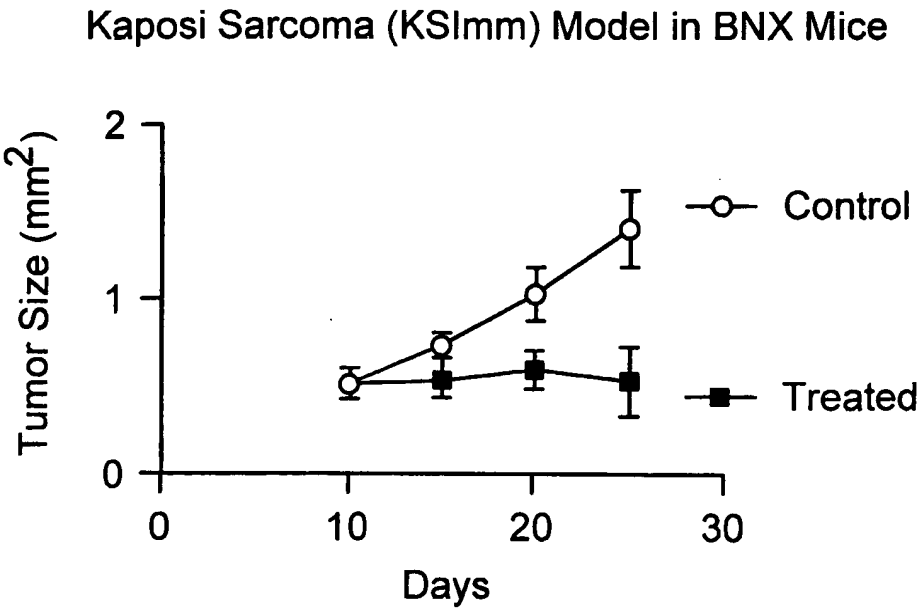


FIG. 26A

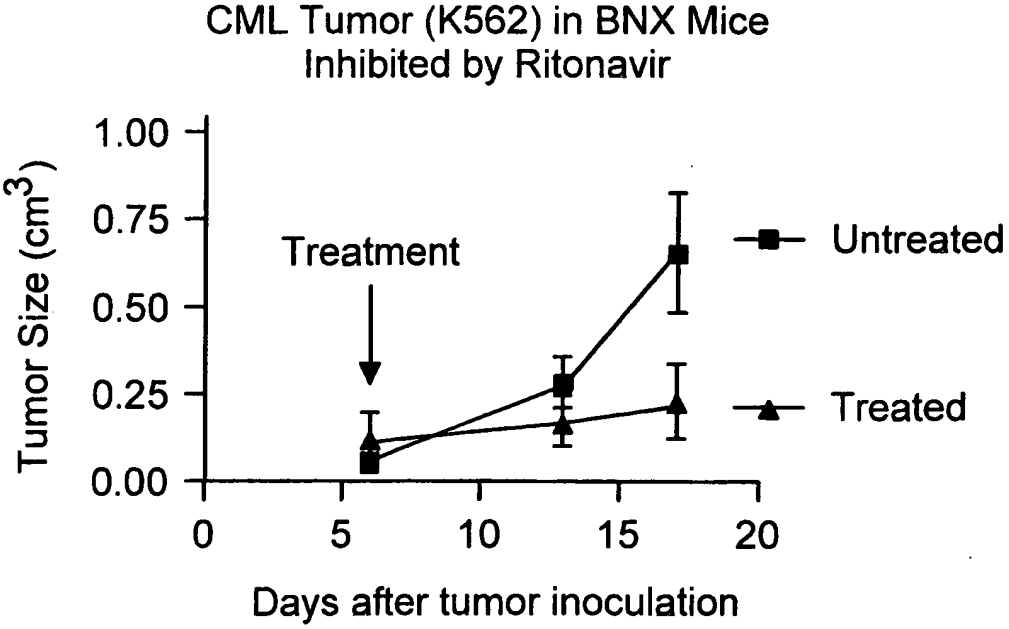


FIG. 26B

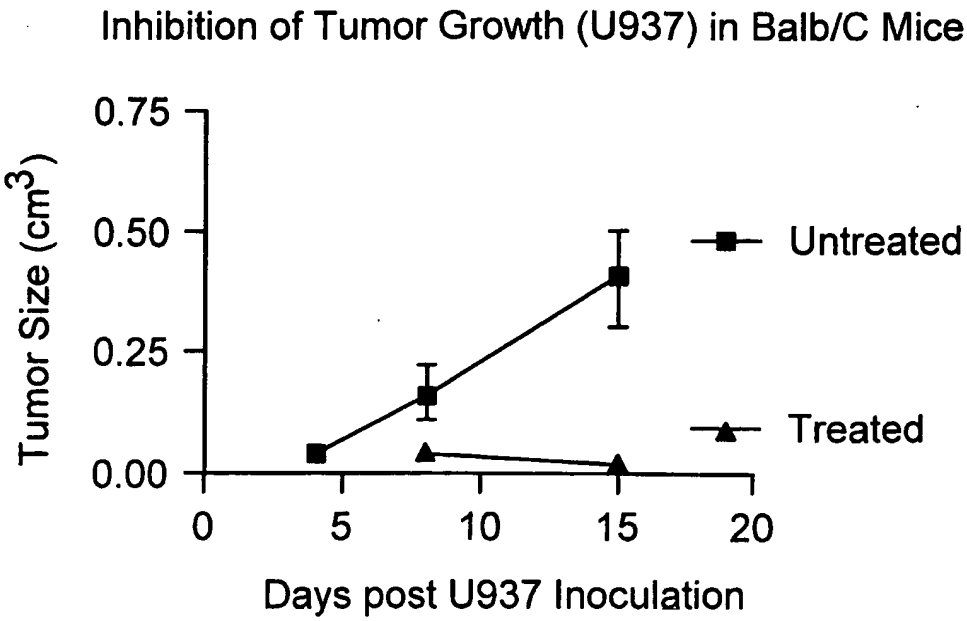


FIG. 27A

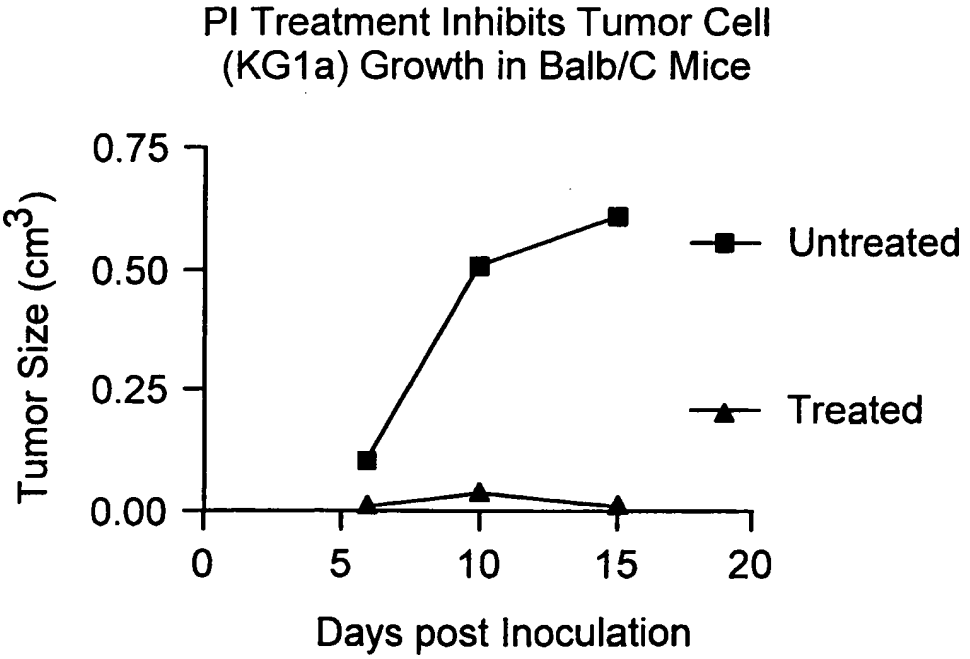


FIG. 27B

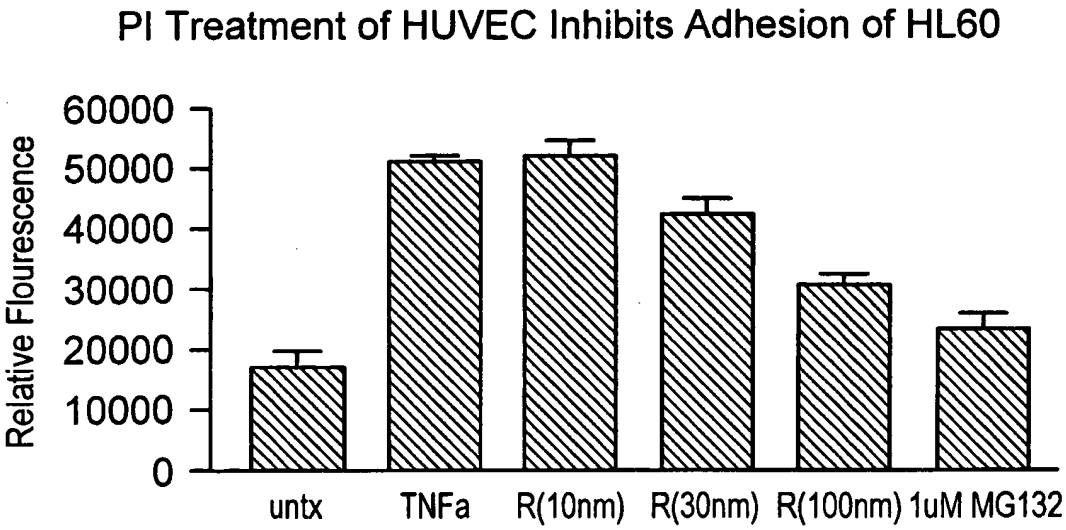

PI Treatment of HUVEC Inhibits Adhesion of HL60
Relative Flourescence
60000
50000
40000
30000
20000
10000
0
untx
TNFa
R(10nm)
R(30nm)
R(100nm)
1uM MG132

FIG. 28

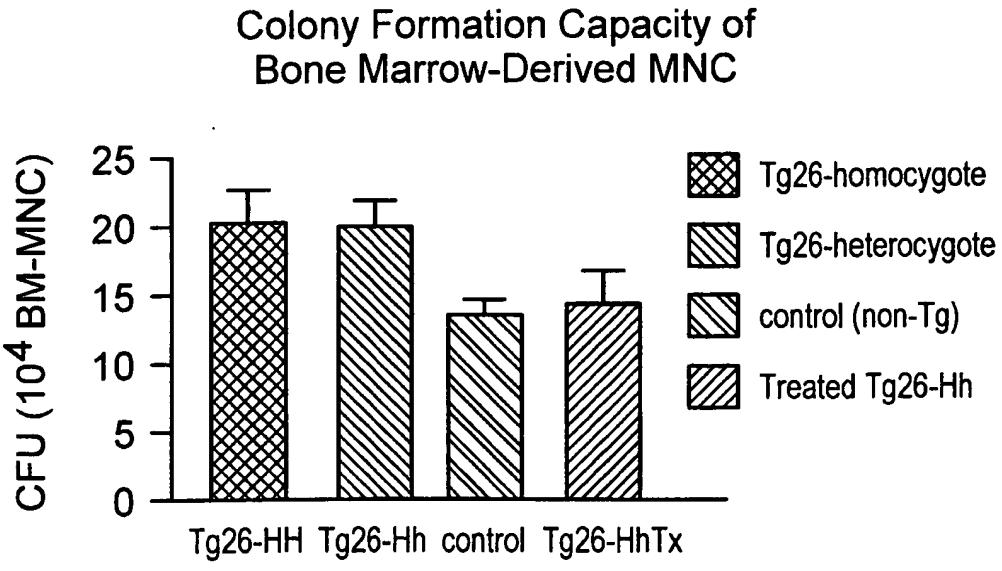

Colony Formation Capacity of
Bone Marrow-Derived MNC
CFU (10^4 BM-MNC)
25
20
15
10
5
0
Tg26-HH
Tg26-Hh
control
Tg26-HhTx
Tg26-homocygote
Tg26-heterocygote
control (non-Tg)
Treated Tg26-Hh

FIG. 29

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28548

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01N 37/18, 43/04; A61K 31/70; 38/00, 38/48
US CL : 424/94.63; 514/2, 42, 43, and 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.63; 514/2, 42, 43, and 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, USPATFUL, Derwint

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,624,934 A (HORNBACK et al.) 29 April 1997(29.04.97), see entire document.	1-4, 10, and 12-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 FEBRUARY 2000	Date of mailing of the international search report 25 APR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY STUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/28548

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 10, and 12-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
T/US99/28548

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be searched, the appropriate additional search fees must be paid. The species are as follows:

Applicant must choose one of the following protease inhibitors:
HIV protease inhibitor
proteosome inhibitor
serine inhibitor
cysteine inhibitor,

and choose one of the following disorders:
autoimmune
cancer
infections disease
allergic disorder
immune dysregulation
wasting syndrome
ischemia
inflammatory condition
trauma shock transplantation
burn
infection
- parasite
- bacterial
- fungal
- retrovirus
- hepatitis
- herpes
- influenza
- papilloma
- HIV-2

malnutrition.

The following claims are generic: 1 and 2

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: Each of the species set forth is a distinct. The chemical compounds are each different types of protease inhibitors and each of the diseases is different from the other and has different underlying causes and outcomes. The examined invention will be HIV protease inhibitor and treating cancer, claims 1-4, 10, and 12-20.